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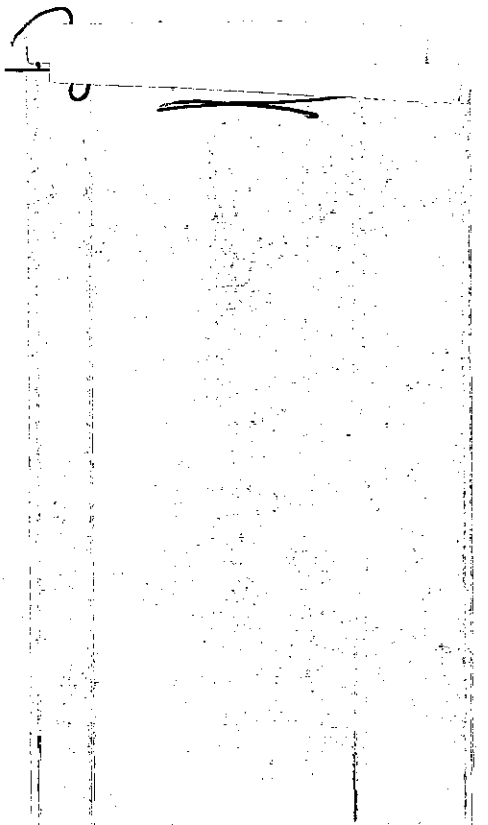

THE CHEMISTRY OF VIOMYCIN AND THE SYNTHESIS OF SOME MODEL PYRROLIDINE AMINO ACIDS

A THESIS

Presented to

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by

Riyad Farid Nassar

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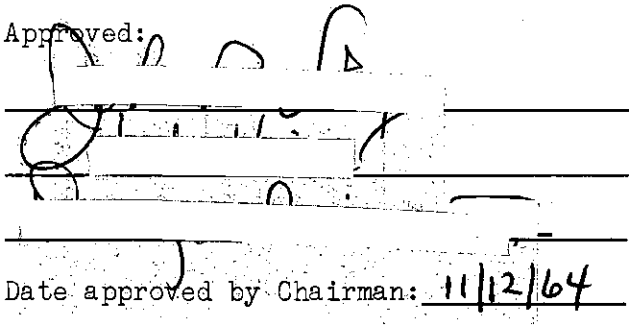
THE CHEMISTRY OF VIOMYCIN AND THE SYNTHESIS OF SOME MODEL PYRROLIDINE AMINO ACIDS

Approved: 

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ACKNOWLEDGMENTS

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The author is indebted to his parents and especially to his wife for the patience, understanding, and encouragement they have so unselfishly given throughout the entire period of study.

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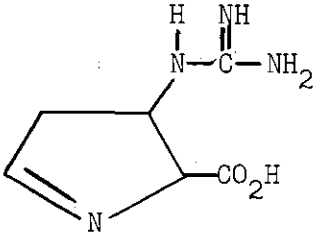
SUMMARY

The purpose of this research was to establish the correct structure for viomycidine, a hydrolysis product of the antibiotic viomycin, and to determine as many of the structural features of viomycin as possible. The ultimate goal of investigations of the chemistry of viomycin is to determine the structure of viomycin in complete detail.

Viomycidine has been previously reported to have the formula $C_6H_{10}N_4O_2$ and to contain no $\underline{C}$-CH_3, $\underline{O}$-CH_3, $\underline{N}$-CH_3, or primary amino groups. At the beginning of this research the three structures, I, II, and III were suggested for the structure of viomycidine on the basis of the



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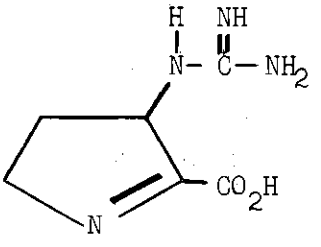

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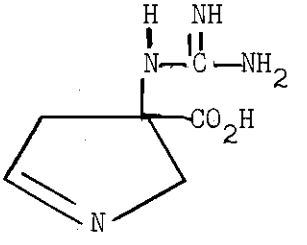


III

formula, the optical activity, the $\underline{pK}_a$ values, the presence of a monosubstituted guanidine group (Sakaguchi test), the nuclear magnetic resonance and infrared spectra, the presence of a reducible double bond in a Δ^1-pyrroline moiety, and other chemical data. Viomycidine was also characterized by two highly crystalline derivatives, the 2,4-dinitrophenyl- and the acetyl-derivative.

The presence of a reducible double bond in viomycidine and the fact that a guanidine group can be hydrolyzed by base to an amino group

made it possible that a pyrrolidine amino acid could be obtained from hydrogenation and basic hydrolysis of viomycidine.

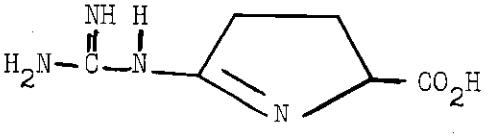
Accordingly, several amino acids were synthesized in order to be compared with the amino acid that might be obtained from viomycidine. cis-4-Aminopyrrolidine-2-carboxylic acid was prepared by the catalytic reduction of 4-nitropyrrole-2-carboxylic acid. The amino acid obtained was characterized by the N,N'-dibenzoyl and the bis-2,4-dinitrophenyl derivatives and by the bis-2,4-dinitro-1-naphthol-7-sulfonate salt. cis-3-Aminopyrrolidine-2-carboxylic acid was prepared by the catalytic reduction of 3-nitropyrrole-2-carboxylic acid. The latter was obtained as a mixture of 3- and 4-nitro isomers by the nitration of pyrocoll. The mixture was purified and the pure 3-nitropyrrole-2-carboxylic acid was reduced to the pyrrolidine amino acid. This amino acid was characterized by the dehydro-N,N'-dibenzoyl derivative and the bis-2,4-dinitro-1-naphthol-7-sulfonate salt.

As a consequence of an attempted synthesis of viomycidine (structure III), another amino acid, 4-aminopyrrolidine-2,4-dicarboxylic acid was synthesized by the Strecker reaction with 1,2-dicarbethoxy-4-pyrrolidone followed by acid hydrolysis. This new amino acid was characterized by the bis-2,4-dinitrophenyl derivative and by the bis-2,4-dinitro-1-naphthol-7-sulfonate salt.

4-Aminopyrrolidine-2,4-dicarboxylic acid and cis-4-aminopyrrolidine-2-carboxylic acid were tested biologically and were found to have no antiviral or antibacterial activity.

Structure II (3-guanido-Δ^1-pyrroline-2-carboxylic acid) had been proposed as the structure of viomycidine. However, the positions of the

guanidine group and the double bond were not established with certainty. Δ^1-Pyrrolines usually give N-acetyl-Δ^2-pyrrolines on acetylation. When acetylviomycidine was ozonized, followed by oxidative work-up and acid hydrolysis, DL-aspartic acid and guanidine were produced as the only observable products. If structure II had been correct, guanidine and β-alanine would have been produced. As no β-alanine was found, structure II is excluded and a new structure, IV, 2-guanido-Δ^1-pyrroline-



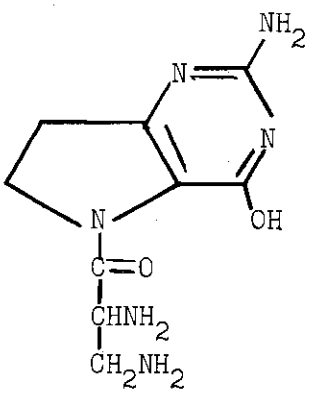
IV

5-carboxylic acid, is proposed for viomycidine. The formation of racemic aspartic acid is attributed to the racemization of the asymmetric center of viomycidine by hot pyridine-acetic anhydride. As viomycidine is more dextrorotatory in acid solution than in water, application of the Clough-Lutz-Jirgensons rule suggests the L (or R) configuration for the asymmetric center present.

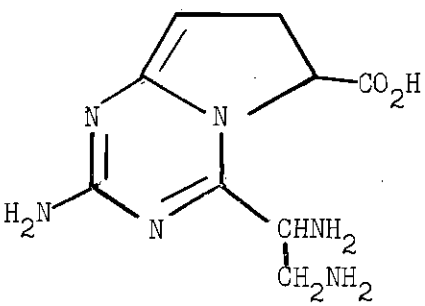
A report from another laboratory described the isolation of a dipeptide that contained only viomycidine and α,β-diaminopropionic acid. Based on the properties of the dipeptide, and incorporating structure II for viomycidine, structure V was proposed for the dipeptide. Using the proposed structure for viomycidine (IV), a new structure (VI) is proposed for the dipeptide.

Previous investigations of viomycin have shown that the barium hydroxide hydrolysate of the antibiotic revealed by paper chromatography,

beside the usual amino acids obtained from the acid hydrolysis, except



V



VI

viomycidine, a ninhydrin positive compound which was tentatively identified as alanine. The barium hydroxide hydrolysis was repeated and the unknown compound was isolated and purified by paper and carbon chromatography. The unknown was identified as DL-alanine by paper chromatography, optical rotation, infrared and nuclear magnetic resonance spectra, and by the 2,4-dinitrophenyl derivative.

When the barium hydroxide hydrolysate was acidified and distilled it liberated acetic acid in 20 per cent yield. The residue, when tested, did not show the presence of oxalic acid, but gave pyrrole-2-carboxylic acid in three per cent yield. The antibiotic was found to be unaffected by the enzyme pronase.

Viomycin, viomycidine, urea, serine, and α,β-diaminopropionic acid were hydrolyzed with barium hydroxide and the amount of ammonia liberated was determined versus time. Serine and α,β-diaminopropionic acid released a negligible amount of ammonia in four days. Viomycin and viomycidine gave about three moles each in three days and urea was

completely hydrolyzed during the same period of time. Analysis of the results at various reaction times revealed that viomycin released, relatively selectively, about one mole of ammonia in excess of that released from urea in the first twenty hours of hydrolysis. This suggested the transformation of a "guanidine" group to a "ureido" group in viomycin during that time.

Analysis of the acid hydrolysate of viomycin indicated a ratio of 1.00 mole of urea, 0.110 mole of ammonia and 0.145 mole of viomycidine. Urea released 0.073 mole of ammonia under the same hydrolysis condition. This indicated, in agreement with the elemental analysis of viomycin, that ammonia is an artifact in the hydrolysate and viomycin probably has the molecular formula $C_{23}H_{38}N_{12}O_9$.

CHAPTER I

INTRODUCTION

Viomycin

The antibiotic viomycin was isolated from cultures of Streptomyces puniceus and Streptomyces floridae simultaneously in the laboratories of Charles Pfizer and Company (1) and Parke, Davis and Company (2) in 1950. Early biological studies indicated that viomycin was useful against tuberculosis (1). Later studies with humans in advanced stages of tuberculosis over periods of two to ten weeks indicated that viomycin caused kidney damage, vestibular dysfunction, electrolyte imbalance, and hypersensitivity (3). However, viomycin is still used clinically when the tuberculosis microorganism becomes resistant to streptomycin.

Several salts of viomycin have been prepared: the chloride, the picrate, the reineckate, and the sulfate. Analytical data on these salts were consistent with an empirical formula of $C_{18}C_{31-33}N_9O_8$ for the free base (3,4). Later, a diaphragm-cell diffusion technique, using the sulfate salt, indicated a molecular weight of 772 for the sulfate salt, which, together with analytical data and chemical evidence, suggested a molecular formula of $C_{25}H_{44}N_{12}O_{11} \cdot 3/2\ H_2SO_4$ (Mol. wt. 836) (5). The sulfate melts at 252° with decomposition and has a specific rotation of -39.8° in water (2).

The antibiotic is a strong base, and has pK_a* values of 8.3, 10.3, and 12.2 in water. The pK_a values of 8.3 and 10.3 are due to amino groups and that of 12.2 is due to a guanidine function. The absence of a low pK_a value indicated that viomycin does not have a free carboxylic group (5). Van Slyke primary amino nitrogen value indicated that 1.22 primary amino groups in viomycin react in 2.5 minutes, 1.98 groups react in 15 minutes, and 2.16 groups react in an hour (assumed molecular weight, 836) (6).

Viomycin exhibits one strong absorption peak in the ultraviolet region. The position and intensity of this peak depend on the pH of the solution. The peak occurs at 268 mμ (ε, 23,300) in 0.1 N hydrochloric acid, at 268.5 mμ (ε, 22,900) in neutral solution, and at 282.5 mμ (ε, 14,600) in 0.1 N sodium hydroxide. Determination of the ultraviolet spectra of viomycin in solutions of pH 10, 11, 12, 13, and 14, respectively, indicated two isosbestic points at 235 and 281 mμ. This indicated that one dissociating group was involved with the chromophore (5,7). Evaluation of the spectra showed that the dissociating group has a pK_a value of 12.4, which indicated that the guanidine group is involved in the chromophore (5). Catalytic hydrogenation of viomycin did not change the ultraviolet spectrum. This showed that the chromophore has no easily reducible group (5).

A positive biuret test and the resistance to mild acid hydrolysis

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* $pK_a = -\log (H^+)(A^n)/HA^{n+1})$, where either A^n or HA^{n+1} can be the compound in question or the compound in a different state of protonation.

indicated that viomycin has a peptide function (4). Hydrolysis of viomycin sulfate with 1 N hydrochloric acid for eleven days at 37° reduced the microbiological activity to 25 per cent of the original value, but did not release appreciable amounts of amino acids.

Viomycin gave positive Sakaguchi and ninhydrin tests, but negative Benedict's, Fehling's, and maltol tests (1,2,6). These results indicated the presence of guanidine and amino groups, but no maltol-forming function. The presence of a carbohydrate moiety was also made unlikely on the basis of color tests and failure of reaction with periodic acid (1,2).

Hydrolysis of viomycin with boiling 6 N hydrochloric acid or 6 N sulfuric acid destroyed the ultraviolet chromophore and released amino acids as shown by the data in Table 1 (4,5). The relative amounts of the

Table 1. Evidence for the Release of Amino Acids from Viomycin[a] by 6 N Hydrochloric Acid Hydrolysis.

	Ninhydrin CO_2	Van Slyke Amino N	Reduction of Periodic Acid
Viomycin	0.0	1.57	0.15 (15 minutes at pH 4.55)
Hydrolysate	3.7	5.8	3.0 (10 minutes at pH 4.7)

a. All values are expressed as groups per mole of viomycin (assumed molecular weight 688).

compounds released after hydrolysis of viomycin with 6 N hydrochloric acid at 100° for six and sixteen hours are shown in Table 2.

Urea and the amino acids were separated from the guanido compounds in the hydrolysate by ion exchange treatment (4). Urea was isolated as

the dixanthydryl derivative. L-Serine was identified by optical rotation, analysis, and by preparation of the N-2,4-dinitrophenyl derivative (4). L-α,β-Diaminopropionic acid was identified as the monohydrochloride salt by analysis, van Slyke amino nitrogen, and ninhydrin carbon dioxide. Its optical rotation and the optical rotation of the N,N'-dibenzoyl derivative indicated an L-configuration (5). L-β-Lysine was isolated and found to

Table 2. Compounds Released by Acid Hydrolysis of Viomycin[a]

Compound	16 Hours (4)	6 Hours (5)
Carbon Dioxide	0.88	0.50
Ammonia	0.92[b]	0.70[b]
Urea	0.40[c]	0.74[d]
L-α,β-Diaminopropionic Acid	0.14[e]	0.42[e]
L-β-Lysine	0.44[e]	0.90[e]
L-Serine	1.30[e]	----
Viomycidine	0.16[e]	0.21[e]

a. All values expressed as moles per mole of viomycin (assumed molecular weight 688).

b. Ammonia was determined as volatile base by aeration from basic solution into boric acid.

c. Urea was determined by conversion to ammonia by urease followed by determination of the resulting ammonia.

d. Urea was determined by the biacetylmonoxide method of Ormsby (8).

e. The amounts of the amino acids were determined by isolation.

be identical to L-3,6-diaminohexanoic acid (4). The mixture of guanido compounds in the hydrolysate was found to contain a major component which was isolated in crystalline form and has been named viomycidine (9).

Hydrolysis of viomycin with 0.43 N barium hydroxide or 0.5 N lithium hydroxide at 95° for three days gave ammonia, carbon dioxide, β-lysine, serine, α,β-diaminopropionic and a substance that was tentatively identified as alanine. These amino acids components of the hydrolysis product were identified by two-dimensional paper chromatography (5).

Hydrolysis of viomycin with 6 N hydrochloric acid at room temperature released urea, serine, and β-lysine during the first day. Paper chromatography indicated that the amount of serine released increased up to ten days and the amount of β-lysine released increased up to the fourth day. After about eleven days a complex pattern of ninhydrin positive spots were observed. No discrete spot corresponding to α,β-diaminopropionic acid or to viomycidine was observed (6).

Viomycin was oxidized rapidly with potassium permanganate solution. The oxidation resulted in the destruction of the ultraviolet chromophore and the consumption of four moles of permanganate per three moles of viomycin (assumed molecular weight of viomycin sulfate 774). No fragmentation of viomycin took place and no carboxylic acid groups were produced in the oxidation. Paper chromatography indicated that the 6 N acid hydrolysate of the oxidation product of viomycin contained the same compounds as the 6 N acid hydrolysate of viomycin sulfate (5).

When the antibiotic in cold aqueous acetic acid was treated with ozone and then with hydrogen peroxide, the ultraviolet chromophore was also destroyed. Papergram analysis of the ozonolysis product indicated

only one ninhydrin-positive compound. This indicated that the site of oxidation is in a cyclic residue in the viomycin molecule. Hydrolysis of the ozonolysis product with 6 N hydrochloric acid at 95° for six hours released serine, β-lysine, α,β-diaminopropionic acid, viomycidine, guanidine, and an unknown guanidine compound (6,10). The release of guanidine suggested the presence of an ethylenic guanidine unit in viomycin (5,6).

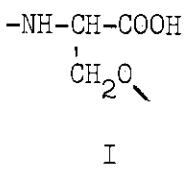
The 2,4-dinitrophenyl derivative of viomycin was prepared and hydrolyzed with concentrated hydrochloric acid at 95° in a pressure bottle for 20 hours. The only 2,4-dinitrophenyl derivative obtained from the hydrolysate was bis-2,4-dinitrophenyl-L-β-lysine (6). This indicated that the two free amino groups of viomycin are those of the L-β-lysyl residue.

Hydrolysis of viomycin with 0.1 N hydrochloric acid at 95° for six hours resulted in the destruction of the ultraviolet chromophore and produced one mole of urea. The remaining polypeptide fragment, named desureaviomycin, was isolated and characterized (5,6). Desureaviomycin was found to have properties very similar to those of viomycin. Hydrolysis of desureaviomycin with 6 N hydrochloric acid at 95° for six hours gave all the components of viomycin hydrolysate except urea. Hydrazinolysis of desureaviomycin indicated the polypeptide had a free seryl carboxyl group (6). Hydrolysis of bis-2,4-dinitrophenyldesureaviomycin under the same condition used for hydrolysis of bis-2,4-dinitrophenylviomycin gave bis-2,4-dinitrophenyl-L-β-lysine as the only 2,4-dinitrophenyl derivative. This showed that no primary amine groups are released in the hydrolysis of viomycin to desureaviomycin (6).

When desureaviomycin was treated with the enzyme carboxypeptidase

at pH 7 for two days, serine was released and the other product of the hydrolysis, named viomycinic acid, was isolated and characterized (6). Viomycinic acid was found to be unaffected by carboxypeptidase. Preparation and hydrolysis of bis-2,4-dinitrophenylviomycinic acid showed that only the amino groups of the L-β-lysyl residue were free. Acid hydrolysis of viomycinic acid gave all the components of viomycin hydrolysate with the exception of urea (6). Hydrazinolysis of viomycinic acid indicated that the carboxyl group of a seryl residue was free.

These data indicated partial structure I for viomycinic acid, partial structure II for desureaviomycin, and partial structure III for viomycin (6).



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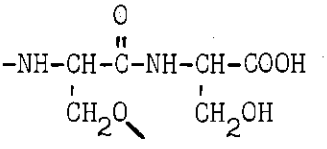

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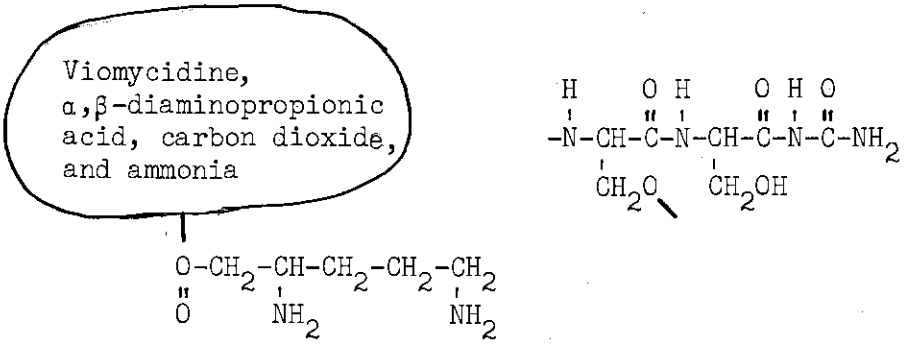


III

In early work, Haskell (4) and his coworkers suggested an empirical formula $C_{18}H_{33}N_9O_8 \cdot 3/2\ H_2SO_4$ (molecular weight, 650.65) for viomycin sulfate

based on elemental analyses. Later, using a diaphragm-cell diffusion technique, Mason (5) determined the molecular weight of viomycin sulfate to be 772 and assigned the molecular formula $C_{25}H_{44}N_{12}O_{11}\cdot 3/2\ H_2SO_4$ (molecular weight 836). However, Mason and later Kellogg (6) noted that the addition of the formulas of the known hydrolysis fragments gave only 20 carbon atoms. The addition gives the following result.

Serine	$C_3H_7N\ O_3$
β-Lysine	$C_6H_{14}N_2O_2$
α,β-Diaminopropionic Acid	$C_3H_8N_2O_2$
Viomycidine	$C_6H_{10}N_4O_2$
Urea	$C\ H_4N_2O$
Carbon Dioxide	$C\quad\ O_2$
Ammonia	H_3N
	$C_{20}H_{46}N_{12}O_{12}$

Subtraction of five moles of water, one for each carboxyl group and one for carbon dioxide gives $C_{20}H_{36}N_{12}O_7\cdot 3/2\ H_2SO_4$ (formula I, molecular weight, 703.73). The isolation of an excess of one mole of serine from the hydrolysate of viomycin and the determination of a serylseryl linkage indicated that viomycin has two serine residues (5-7). If a second serine fragment is added, less a molecule of water, $C_{23}H_{41}N_{13}O_9\cdot 3/2\ H_2SO_4$ (formula II, molecular weight, 790.81) results. Doubt has existed as to the origin of the ammonia that is observed in viomycin hydrolysate because analytical data are in better agreement if the formula has one less nitrogen atom. The ammonia observed could arise by partial acid-catalyzed decomposition of the observed hydrolysis products. If ammonia is an artifact in the hydrolysate, the formula $C_{23}H_{38}N_{12}O_9\cdot 3/2\ H_2SO_4$ (formula III,

molecular weight, 773.77) for viomycin sulfate results (5,6).

Analytical results for carbon, hydrogen, nitrogen, and sulfur that have been reported for viomycin sulfate and the calculated values for the three suggested molecular formulas are shown in Table 3. The ratio of carbon to nitrogen also is given in Table 3 since this value is independent of the dryness of the sample and the stoichiometry of the sulfate salt.

Table 3. Per Cent Composition of Viomycin Sulfate.

	C	H	N	S	C/N
Formula I ($C_{20}H_{36}N_{12}O_7 \cdot 3/2\ H_2SO_4$)	34.14	5.58	23.89	6.83	1.44
Formula II ($C_{23}H_{41}N_{13}O_9 \cdot 3/2\ H_2SO_4$)	34.92	5.61	23.03	6.08	1.52
Formula III ($C_{23}H_{38}N_{12}O_9 \cdot 3/2\ H_2SO_4$)	35.70	5.34	21.72	6.22	1.70
Reported (1)	37.19	5.86	20.61	5.88[a]	1.80
Reported (2)	35.83	5.77	21.08	5.34	1.70
Reported (4)	35.89	5.52	21.15	5.79	1.70
Reported (5)	34.22[b]	5.73[b]	21.85[b]	4.73[b]	1.56[b]
Reported (6)	35.01	6.19	21.01	5.73	1.67
Average of Found	35.71	5.83	21.19	5.51	1.69

a. Determined as sulfate.

b. Not corrected for residue after ignition (1.25 per cent).

The values for the three corresponding formulas for desureaviomycin sulfate (Ia, IIa, and IIIa) are given in Table 4.

Table 4. Per Cent Composition of Desureaviomycin Sulfate.

	C	H	N	S	C/N
Formula Ia ($C_{19}H_{34}N_{10}O_7 \cdot 3/2\ H_2SO_4$, M.W., 661.58)	34.48	5.63	21.17	7.27	1.63
Formula IIa ($C_{22}H_{39}N_{11}O_9 \cdot 3/2\ H_2SO_4$, M.W., 748.76)	35.29	5.65	20.58	6.42	1.71
Formula IIIa ($C_{22}H_{36}N_{10}O_9 \cdot 3/2\ H_2SO_4$, M.W., 731.73)	36.11	5.37	19.14	6.57	1.89
Reported (6)	37.21	5.50	18.67	5.63	1.99

The values for the three formulas (Ib, IIb, and IIIb) suggested for viomycinic acid sulfate are given in Table 5.

Table 5. Per Cent Composition of Viomycinic Acid Sulfate.

	C	H	N	S	C/N
Formula Ib ($C_{16}H_{29}N_9O_5 \cdot 3/2\ H_2SO_4$, M.W., 574.60)	33.45	5.61	21.94	8.37	1.52
Formula IIb ($C_{19}H_{34}N_{10}O_7 \cdot 3/2\ H_2SO_4$, M.W., 661.68)	34.49	5.64	21.17	7.27	1.63
Formula IIIb ($C_{19}H_{31}N_9O_7 \cdot 3/2\ H_2SO_4$, M.W., 644.65)	35.40	5.32	19.56	7.46	1.81
Reported (6)	35.68	5.54	21.64	7.05	1.65

Viomycidine

The acid hydrolysate of viomycin was found to contain a mixture of strongly basic compounds that gave positive Sakaguchi tests (4,5). These basic compounds were separated from the other hydrolysis products by ion-exchange treatment. After ammonia was removed, the basic fraction was purified by carbon chromatography. A crystalline compound that was named viomycidine was obtained (9). Viomycidine was assigned the formula $C_6H_{10}N_4O_2$ on the basis of analysis of its monohydrochloride salt (9). The crystalline monohydrochloride melted with decomposition at 200-208° and was optically active. The specific rotation varied with pH. It was -21.2° in 2 N hydrochloric acid, -83.2° in water, and -155.8° in 1 N sodium hydroxide (9). Preparation of the 2,4-dinitrophenyl derivative of viomycidine gave a yellow crystalline compound that melted at 171.5-172.6° with decomposition. Elemental analysis gave a formula of $C_{12}H_{12}N_6O_6 \cdot 2H_2O$ for this derivative. This derivative gave a pink color with the Weber reagent but a negative test with the ninhydrin reagent. The acetyl derivative, prepared using aqueous ethanolic acetic anhydride, was a white crystalline solid that melted at 256-257° with decomposition. The derivative had an optical rotation of +41° in water and an empirical formula of $C_8H_{12}N_4O_3$ (11). The compound gave an orange color with the Weber reagent but negative tests with the ninhydrin, o-aminobenzaldehyde, Sakaguchi, and Benedict-Behre reagents (11).

Viomycidine has no N-methyl, O-methyl, C-methyl or primary amino groups. It has pK_a values of 2.8, 5.87, and 13.4 in 66 per cent N,N-dimethylformamide and 5.50 and 12.6 in water (9). The carboxylic acid function was too strong to be accurately determined in water by the method

used but was estimated to be 1.3 (11). The pK_a value 12.6 in water and 13.4 in 66 per cent dimethylformamide is consistent with the presence of a guanidine group. The group whose pK_a value is 5.50 in water has a differential ultraviolet spectrum. A determination using pH 9.80 in one cell and pH 3.82 in the other shows that the weakly basic group absorbs at 212 mμ and has an extinction coefficient of 2,530. This indicated a tertiary amino group. A determination at pH 9 versus pH 13.1 shows λ_{max} 222 mμ, ε 1690 (9,11,12).

Viomycidine gave a pink color with the Sakaguchi reagent, a result indicative of a monosubstituted guanidine group, and a pink-purple color with the ninhydrin reagent. It gave a negative result with Tollen's reagent and no color change was produced with the Benedict-Behre reagent. When heated under the conditions for the transformation of creatine into creatinine, it did not give a positive test with Benedict-Behre reagent but still gave a positive test with the Sakaguchi reagent. This showed that no cyclization to the creatinine-type linkage had occurred. The compound gave a negative ferric chloride test, which indicated the absence of an enolic group (9).

The infrared spectrum of viomycidine hydrochloride showed a large number of absorptions. The only absorptions which were structurally characterized were those for N-H stretching, C=N stretching, C-H stretching, CH_2 deformation, and carboxylate anion vibrations (9).

The formula of viomycidine indicated four rings and/or double bonds. The carboxylic acid and guanidine groups account for two double bonds and the fact that viomycidine absorbed one mole of hydrogen in the presence of platinum and acetic acid indicated that viomycidine has a

cyclic structure and one double bond (9,11).

Viomycidine gave a yellow color when treated with the o-aminobenzaldehyde reagent, a typical test for imines and Δ^1-pyrrolines. This structural unit would account for the ring, the double bond, the tertiary amino (cyclic imine) group, and the weakly basic group of viomycidine (11).

When viomycidine was heated with concentrated hydrochloric acid for six days it was partially converted into at least five other ninhydrin positive compounds, some of which gave positive Weber reactions. An appreciable amount of viomycidine remained after this length of time (9). This might account for the low yield of viomycidine and the occurrence of other minor guanido compounds produced in the hydrolysis of viomycin. The other guanido compounds obtained from the acid hydrolysis of viomycin have not been isolated in pure form.

Oxidation of viomycidine with nitric acid gave guanidine. No other Weber or ninhydrin positive spots appeared on the papergram. Oxidation with hot acidic potassium permanganate gave a number of compounds, none of which could be isolated or identified (11).

When acetylviomycidine was treated with ozone followed by hydrogen peroxide and acid, the product obtained consisted of a mixture of four ninhydrin- and Weber-positive substances, but showed no viomycidine by paper chromatography. Since viomycidine is reasonably stable to acid (9), and carboxyl and guanidine groups are not attacked by ozone, the absence of viomycidine from the product indicated that acetyl viomycidine, and hence viomycidine, has a double bond (11).

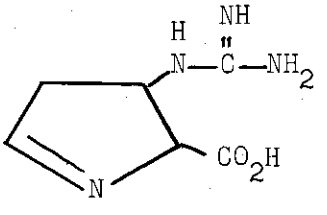
Barium hydroxide hydrolysis of viomycidine at 95° for 77 hours resulted in the evolution of 2.71 moles of ammonia and the formation of

0.45 moles of barium carbonate (13). Pyrrole-2-carboxylic acid was also isolated from the hydrolysate and identified. This result showed that viomycidine has five carbon atoms in a straight chain with the carboxylic acid group at one end of that chain (11).

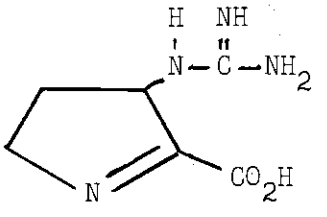
When viomycidine was hydrolyzed with hot concentrated sodium hydroxide at 100°, 2-aminopyrimidine, glycine, and pyrrole-2-carboxylic acid were obtained and identified (11,13).

The nuclear magnetic resonance spectrum of viomycidine hydrochloride in deuterium oxide solution revealed absorption at 7.43 τ (two protons, triplet, J = 2.1 cps), at 5.38 τ (two protons, closely spaced multiplet), and at 4.37 τ (one proton, triplet, J = 2.2 cps) (11,14). The spectrum in trifluoroacetic acid solution revealed the same proton ratio (2:2:1) for the hydrogens bonded to carbon. In addition, three more absorption peaks were observed at 3.00 τ (two protons), 1.98 τ (one proton) and 1.48 τ (one proton); none of these peaks was split (11).

On the basis of the physical and chemical data mentioned above, two possible structures were suggested for viomycidine, 4-guanido-Δ^1-pyrroline-5-carboxylic acid (IV), and 3-guanido-Δ^1-pyrroline-2-carboxylic acid (V) (11). Structure IV was rejected because of the absence of a low-



IV



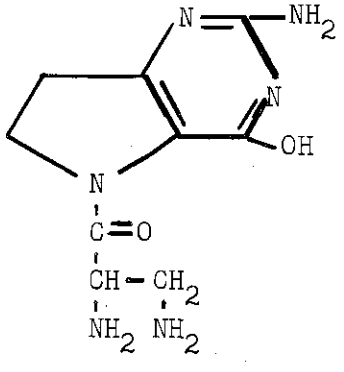
V

field proton in the nuclear magnetic resonance spectrum of viomycidine. To estimate the absorption positions of the protons of viomycidine, the

nuclear magnetic resonance spectra of various model compounds were used. The estimates of the absorption positions for structure V gave better comparisons with the observed value than the estimates for structure IV. On the basis of these results structure V was suggested as the structure of viomycidine (11).

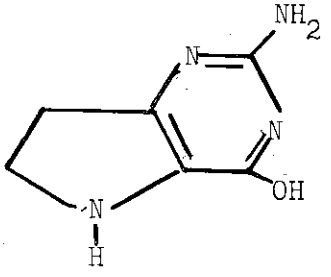
In a recent paper, A. W. Johnson (15) reported the isolation of a dipeptide from the acid hydrolysate of viomycin. The dipeptide has an ultraviolet spectrum similar to that of viomycin (an absorption at 275 mμ (ε 5100) at pH 1 and at 295 mμ (ε 3600) at pH 10). It gave positive ninhydrin and Sakaguchi reactions, had pK_a values of 9.9 and 11.2 and was not hydrogenated in the presence of palladium or carbon. Hydrolysis of the dipeptide with boiling concentrated hydrochloric acid gave a α,β-diaminopropionic acid and viomycidine in a 1:1 molar ration. Treatment of the dipeptide with 2,4-dinitrofluorobenzene gave a 2,4-dinitrophenyl derivative which on hydrolysis gave α,β-bis-2,4-dinitrophenylaminopropionic acid. Oxidation of the dipeptide with dilute aqueous permanganate gave guanidine and a new peptide which upon hydrolysis gave α,β-diaminopropionic acid.

In a previous report from this laboratory structure V was suggested for viomycidine (16). Johnson accepted this proposed structure and with the above data he postulated structure VI for his dipeptide.



VI

On the basis of the similarity of the ultraviolet spectral data of viomycin and the dipeptide, Johnson also suggested that viomycidine is present in the antibiotic as the cyclic structure VII, a derivative of 6,7-dihydro-5H-pyrrolo[3,2-d]pyrimidine (15).



VII

CHAPTER II

EXPERIMENTAL

Apparatus and Techniques

Ion exchange resins used in this work were regenerated and used as described previously (9). The following abbreviations are used: IR-45(OH^-) and IR-45(Cl^-) for Amberlite anion exchange resin 45 in the hydroxyl and the chloride phase, respectively, IR-400(OH^-) for Amberlite anion exchange resin 400 in the hydroxyl phase, and IRC-50(H^+) for Amberlite cation exchange resin 50 in the hydrogen phase. When the ion exchange resins were used in columns, a minimum of a six-fold excess of resin was used. When resins were used in batches, the sample was eluted by washing the resin with about five times as much water as the volume of the resin used. When the resins were used in columns, the sample was eluted with at least two column volumes of water.

Apparatus and techniques used in paper chromatography include those previously described (9). In addition, descending chromatography was carried out by placing the solvent in a glass dish on a tripod. The papergram was supported by placing a heavy glass rod or a flask full of water on its upper end in the middle of the glass dish. The papergram was allowed to hang down the side of the dish over a glass beaker receiver. The tripod was placed on a glass plate and covered with a bell jar to provide an atmosphere saturated with the solvent vapor. The solvent systems which were employed and their abbreviations are: t-butyl alcohol-acetic

acid-water 2:1:1 (v/v) (BAW), n-propyl alcohol-acetic acid-water 10:1:9 (v/v) (PAW), phenol-water 4:1 (v/v) (PW), n-butyl alcohol-acetic acid-water 5:4:1 (v/v) (n-BAW), water, and isobutyric acid. Spray reagents which were most frequently used were ninhydrin, Weber, and Erhlich. Their preparation and interpretation of results are as given previously (17,18).

Thin layer chromatography (TLC) was used during this work. The absorbant used was silica gel G prepared on glass plates 20 cm. x 20 cm., 20 cm. x 10 cm., and 20 cm. x 5 cm. in size according to the directions of Stahl using a Stahl spreader (Model S) (19). A 0.25 mm. thick plate was used in qualitative analysis and a 1 mm. thick plate in preparative work. For preparative work, the sample was applied, using a syringe, in a streak along the width of the plate about 3 cm. from the bottom. About 2 mg. of substance per 1 cm. plate width was used. After development in the appropriate solvent and drying, the bands were collected, using a microvacuum cleaner (19). The solvent systems which were used for TLC are: t-butyl alcohol-methanol-3 N ammonia 3:1:1 (v/v) (BMA), t-butyl alcohol-5 N ammonia (v/v) (BA), methanol-4 N ammonia 4:1 (v/v) (MA), BAW, PAW, ethyl acetate, n-butanol, methanol, and ethanol. Ninhydrin and Nessler's reagents were used routinely for color tests. The ninhydrin reagent was the same as the spray reagent used in paper chromatography and its use has been previously described (9). The Nessler's reagent was prepared and used to detect ammonia as described previously (9).

Pretreated (3% oleic acid) Darco G-60 (Atlas Powder Company) was prepared and mixed with acid-washed celite (Johns-Manville Corporation) as described previously (9). The carbon columns so prepared were washed

with water until the wash water gave a negative test for alcohol with the ceric nitrate reagent (20). About 100 g. of charcoal-celite mixture was used per gram of sample and the columns were prepared as described previously (9).

All melting points were determined, using a Kofler micro hot stage. Optical rotations were determined using a Bellingham and Stanley Model No. 397619 polarimeter; the sodium D line was used as a light source. All ultraviolet spectra were determined using a Cary Model 14 recording spectrophotometer. A Perkin Elmer Model 137 recording spectrophotometer was used to obtain infrared spectra. Potassium bromide pellets were prepared by grinding about 1.0 mg. of the sample in about 140 mg. of potassium bromide; the pellet was prepared at 2500 psig.

A Varian Model A-60 spectrometer was used to obtain nuclear magnetic resonance spectra. The following reference compounds and their abbreviations are used: tetramethylsilane internal standard (TMS), tetramethylsilane (5% in carbon tetrachloride) external (TMS, external standard), and sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS).

A Research Specialties Company Model 1205 fraction collector was used in all column chromatographic separations. Microanalyses were performed by Galbraith Laboratories (Knoxville, Tennessee), Huffman Laboratories (Wheatridge, Colorado), and A. Bernhardt Microanalytical Laboratory (Mülheim, West Germany).

A Welsbach Model T-23 ozone generator was used in all ozonolyses. Ozone was generated at 120 volts and a pressure of 6 psig.

Unless otherwise indicated, hydrogenations were carried out in a Parr pressure reaction apparatus. Evaporations were carried out under

reduced pressure using a rotating evaporator.

4-Nitro-2,6-dioxatetrahydrothiapyran-1-oxide

2-Nitro-1,3-propanediol was prepared according to the method of Den Otter (21). The product had m.p. 53-54° (corr.) [lit. (21) m.p. 54°].

To a suspension of 6.0 g. (49.5 mmole) of 2-nitro-1,3-propanediol in 100 ml. of benzene was added 24.0 g. (200 mmole) of thionylchloride. The reaction mixture was boiled under reflux for 20 hr. and then evaporated to dryness *in vacuo* at 35-40°. The light brown solid residue was recrystallized from a mixture of benzene and petroleum ether. The tan needles obtained weighed 6.30 g. (76%) and showed m.p. 97-99° (corr.). The analytical sample was prepared by three additional recrystallizations from benzene-petroleum ether. The sample had m.p. 99-100° (corr.).

Anal. $C_3H_5NO_5S$ (167.15) Calc'd: C, 21.56; H, 3.02; N, 8.38; S, 19.18
Found : C, 22.22; H, 3.54; N, 8.58; S, 19.71

The infrared spectrum of the compound, in chloroform solution, showed, among others, absorptions at 6.40, 7.41, 8.52, and 9.18 μ. The n.m.r. spectrum of 4-nitro-2,6-dioxatetrahydrothiapyran-1-oxide in deuterioacetone (TMS) (*c*, 30%) showed a complex series of absorptions between 4.50 and 5.40 τ.

2-Acetamido-1,3-propanediol

To a solution of 10.0 g. (82.5 mmole) of 2-nitro-1,3-propanediol in 60 ml. of 95% ethanol was added 3.0 g. of 5% platinum on carbon catalyst. The mixture was hydrogenated at room temperature and atmospheric pressure for 2 days. The hydrogen uptake was 98% of the theoretical. The catalyst was filtered and the ethanol was evaporated *in vacuo*. The

viscous oil obtained weighed 7.50 g. (82.4 mmole, 99%). The oil was dissolved in 75 ml. of 50% ethanol and the solution was chilled in ice. To this solution was added 20.0 g. (196.0 mmole) of precooled acetic anhydride. The reaction mixture was kept in the refrigerator overnight. The solution was evaporated *in vacuo* to about 15 ml. at 40-46° and lyopholized. The tan residue was recrystallized from acetone and gave 4.42 g. (40% based on 2-nitro-1,3-propanediol) of white plates, m.p. 85-86° (corr.). Two more recrystallizations from acetone afforded an analytical sample m.p. 85.5-86.5° (corr.) [lit. (22) m.p. 89-90°].

Anal. $C_5H_{11}NO_3$ (133.15) Calc'd: C, 45.09; H, 8.33; N, 10.52
Found : C, 45.38; H, 8.45; N, 10.77

The infrared spectrum of the 2-acetamido-1,3-propanediol in chloroform solution, showed absorptions at 2.90, 3.30, 5.87 (weak), 6.15 and 6.50 μ among others. The n.m.r. spectrum in deuterium oxide (DSS) (c, 20%) showed absorptions at 7.98 τ (3H, singlet) and a complicated series of peaks from 5.9 to 6.6 τ (5H).

Tri-(p-toluenesulfonyl)-2-amino-1,3-propanediol

A solution of 3.0 g. (33.0 mmole) of crude 2-amino-1,3-propanediol in 15 ml. of pyridine was cooled to 0° in an ice bath. To this solution was added portionwise a cooled solution of 66.0 g. (342.0 mmole) of p-toluenesulfonyl chloride in 75 ml. of pyridine. The solution was kept below 5° during the addition. The reaction mixture was allowed to stand in the refrigerator for six days. The deep red solution was poured into an ice-water mixture and neutralized with sodium bicarbonate solution. The aqueous solution was extracted with three 80-ml. portions of chloroform.

The combined chloroform extracts were back extracted with a mixture of hydrochloric acid and ice. The chloroform layer was washed with 100 ml. of cold water, dried over magnesium sulfate, and evaporated to dryness *in vacuo*. The red-brown viscous oil that resulted solidified upon cooling in the refrigerator. Crystallization from benzene afforded 8.90 g. (49% based on 2-nitro-1,3-propanediol) of white solid that had m.p. 139-140° (corr.). An analytical sample was prepared by recrystallizing the compound two more times from benzene. It showed m.p. 139-140° (corr.).

Anal. $C_{24}H_{27}NO_8S_3$ (553.69) Calc'd: C, 52.06; H, 4.92; N, 2.53; S, 17.37
Found : C, 51.65; H, 4.82; N, 2.69; S, 17.78

The infrared spectrum in chloroform solution showed, among others, absorptions at 2.70, 2.91, 7.40, 8.60 μ.

cis-4-Aminopyrrolidine-2-carboxylic Acid Hydrochloride

Preparation and Properties

Freshly recrystallized 4-nitropyrrole-2-carboxylic acid (23) (20.0 g., 129.0 mmole) was hydrogenated at 50 psig at room temperature, using a rhodium catalyst (40.0 g. of five per cent rhodium on alumina), in 200 ml. of redistilled acetic acid. The hydrogen uptake was 78% of the theoretical amount after three days. The catalyst was removed by filtration and the filtrate was treated with IR-45(OH^-) ion exchange resin until the pH rose to 5.0. The supernatant liquid was passed over an IR-45(Cl^-) column containing 400 ml. of resin. The column was washed with one liter of water and the eluate was evaporated to dryness *in vacuo*. The dark brown residue (14.03 g.) was decolorized with activated charcoal and recrystallized from water-ethanol mixture. This gave 7.40 g. (35%) of light tan needles, m.p. 227.5-228.5° (corr.) [lit. (9) m.p. 227-228°].

The amino acid had an R_F value of 0.26 in BAW and gave a yellowish-purple color with the ninhydrin reagent. It had pK_a values of 6.60 and 9.95 in water as shown by potentiometric titration (24). The pK_a value of the acidic group was not determined. The infrared spectrum of the amino acid as a potassium bromide pellet is shown in Fig. 1. The n.m.r. spectrum in deuterium oxide solution (DSS) (c, 40%) showed complex absorptions from 5.48 to 6.05 τ (2.1H), 6.10 to 6.68 τ (1.8H), 6.76 to 7.47 τ (1.42H), and 7.50 to 8.16 τ (1.22H). This spectrum is given as Fig. 2.

Benzoyl Derivative

The benzoyl derivative of cis-4-aminopyrrolidine-2-carboxylic acid hydrochloride was prepared by a general method for the preparation of N-benzoyl amino acids (25). The amino acid (0.80 g., 4.82 mmole) was dissolved in 10 ml. of 2 N sodium hydroxide and the solution was chilled in ice. Alternately there was added to this cold solution 2.0 g. (14.2 mmole) of benzoyl chloride in five portions and 10 ml. of 2 N sodium hydroxide in five portions such that the reaction mixture remained alkaline. The reaction mixture was magnetically stirred throughout the addition and for an additional 20 min. at room temperature. The solution was acidified (congo red) with concentrated hydrochloric acid and the mixture was stored in the refrigerator for two hours. The solid was filtered, washed with ice-water and allowed to dry in air. The dry solid was heated under reflux with 15 ml. of carbon tetrachloride to dissolve the benzoic acid and the mixture was filtered warm. The white residue was recrystallized from an ethanol-water mixture to give 840 mg. (51%) of white solid, m.p. 177-178° (corr.). An analytical sample was prepared by recrystallizing the benzoyl derivative twice more from ethanol-water.

Anal. $C_{19}H_{18}N_2O_4 \cdot 1/2\ H_2O$ (347.38) Calc'd: C, 65.70; H, 5.51; N, 8.06
Found : C, 65.72; H, 5.23; N, 7.78
C, 66.05; H, 5.29; N, 8.05

The infrared spectrum of the dibenzoyl derivative, in chloroform solution, showed strong absorbance at 5.80 μ and 6.07 μ, among others.

bis-2,4-Dinitrophenyl Derivative

The 2,4-dinitrophenyl derivative was prepared by adopting the general method used for amino acids (25). To a solution of 232 mg. (1.39 mmole) of the amino acid and 1.0 g. of sodium bicarbonate in 15 ml. of water was added a solution of 1.00 g. (5.35 mmole) of 2,4-dinitrofluorobenzene in 15 ml. of absolute ethanol. The suspension was allowed to stand at room temperature for two hours with occasional swirling. The ethanol was evaporated *in vacuo* and the reaction mixture was acidified with hydrochloric acid. The yellow precipitate that formed after cooling was collected on a sintered glass funnel, washed with three 5-ml. portions of cold water and then with three 10-ml. portions of benzene. The dry yellow solid weighed 605 mg. (94%), m.p. 228-234° dec. (corr.). The product was recrystallized three times from acetone-water and gave yellow cubic crystals, m.p. 245-248° dec. (corr.).

Anal. $C_{17}H_{14}O_{10}N_6$ (462.35) Calc'd: C, 44.16; H, 3.05; N, 18.17
Found : C, 44.49; H, 3.23; N, 17.62

Flavianate Salt Derivative

To a solution of 0.400 g. (2.40 mmole) of *cis*-4-aminopyrrolidine-2-carboxylic acid hydrochloride was added 15 ml. of a 25% solution of 2,4-dinitro-1-naphthol-7-sulfonic acid (flavianic acid). A yellow precipitate formed within a few minutes. The mixture was heated to boiling

and enough water was added slowly to give a clear solution. After cooling, the yellow solid that formed was collected and recrystallized three more times from water to give 1.18 g. of analytical sample, m.p. 250-255° dec. (corr.).

Anal. $C_{25}H_{22}N_6O_{18}S_2 \cdot 3/2\ H_2O$ (785.66) Calc'd: C, 38.19; H, 3.21; N, 10.70; S, 8.16

Found : C, 38.26; H, 3.61; N, 10.36; S, 8.17

C, 38.18; H, 3.42; N, 10.10; S, 8.12

cis-3-Aminopyrrolidine-2-carboxylic Acid Sulfate

Preparation and Properties

Pyrrole-2-carboxylic acid was prepared by the method of Maxim, Zugravescu and Fulga (26), m.p. 190-191° (corr.) [lit. (26) m.p. 192°]. Pyrocoll was prepared from pyrrole-2-carboxylic acid according to the method of Ciamician and Silber (27), m.p. 267-268° (corr.) [lit. (27) m.p. 267°].

To an ice-cold mixture of 80 ml. of concentrated nitric acid (density 1.42) and 80 ml. of fuming nitric acid (density 1.59) was added 10.0 g. (53.2 mmole) of pyrocoll in six equal portions with continual swirling during30 min. The resulting red solution was allowed to stand at room temperature for 16 hours. The reaction mixture was poured onto 400 ml. of ice-water; the yellow precipitate was collected on a sintered glass funnel and washed with two 20-ml. portions of ice-water. The dry solid was dissolved in 100 ml. of 20% sodium hydroxide solution and the solution was heated on the steam bath for one hour. After cooling, the basic solution was acidified with hydrochloric acid

to pH 1. The acidic solution was extracted with five 150-ml. portions of ether. The combined ether extracts were washed with one 100-ml. portion of cold water, dried over magnesium sulfate, and evaporated to dryness *in vacuo*. This gave 7.1 g. (42%) of yellow solid, m.p. 145-150° (corr.). The n.m.r. spectrum in deuterium oxide and potassium carbonate (*c*, 10%) (DSS, external standard) showed the following absorptions and relative intensities: 2.01 τ (doublet, J = 1.8 cps), 4.8 squares; 2.67 τ (doublet, J = 4.5 cps) and 2.69 τ (doublet, J = 1.8 cps), 13.0 squares; and 3.10 τ (doublet, J = 4.5 cps), 9.1 squares. After three recrystallizations from benzene the yellow solid obtained weighed 4.1 g., m.p. 146-148° (corr.) [lit. (23), m.p. 146°]. The n.m.r. spectrum showed that the three absorptions mentioned above had a ratio of intensities of 1.3, 16.6, and 15.2, respectively.

Hydrogenation of the above 3-nitropyrrole-2-carboxylic acid (5.0 g., 32 mmole) was carried out at 50 psig at room temperature using 10.0 g. of rhodium on alumina catalyst in 60 ml. of acetic acid. The hydrogenation was continued for three days. The catalyst was removed by filtration. The filtrate was evaporated to about 15 ml. *in vacuo* and was neutralized with dry IR-45(OH^-) in absolute ethanol. The ethanol was evaporated, *in vacuo*. Ethanol (200 ml.) was added and the mixture was evaporated to dryness *in vacuo*. The process was repeated four times. The final ethanol mixture gave a negative test with Nessler"s reagent. The amino acid was washed from the resin with water and the solution was passed over an IR-45($SO_4^=$) column containing 80 ml. of resin. The column was washed with 200 ml. of water and the eluate was evaporated to about 30 ml. *in vacuo*. The solution was decolorized with activated charcoal

and evaporated to dryness *in vacuo*. This gave 2.90 g. (50%) of light tan solid. All attempts to crystallize the above solid failed, probably because of its highly hygroscopic nature. A sample of the above crude solid was dried in vacuo at 77° for two days, m.p. 215-220° dec. (corr.).

Anal. $C_5H_{10}N_2O_2 \cdot 1/2\ H_2SO_4$ (179.19) Calc'd: C, 33.51; H, 6.19; N, 15.63; S, 8.95

Found : C, 35.15; H, 6.48; N, 19.52; S, 11.33

The amino acid had R_F values of 0.44 and 0.86 in BAW and water, respectively and gave a purple-brown color with the ninhydrin reagent. The infrared spectrum of the compound as a potassium bromide pellet is given as Fig. 3. The n.m.r. spectrum in deuterium oxide and sulfuric acid solution, pH 1, (DSS) (c, 10%) showed absorptions at 6.31 τ (triplet, 2H, J = 7.5 cps) and two complex absorptions from 6.76 to 7.34 τ (2.08H), and 7.45 to 8.02 τ (2.1H). This spectrum is given as Fig. 4.

Benzoyl Derivative

The benzoyl derivative of *cis*-3-aminopyrrolidine-2-carboxylic acid sulfate was prepared by a general method for the preparation of N-benzoyl amino acids (25). A solution of 500 mg. (2.80 mmole) of the above crude amino acid in 8 ml. of 2 N sodium hydroxide was chilled in an ice-water bath. Alternately there was added to this cold solution 2.00 g. (14.2 mmole) of benzoyl chloride in three portions and 12 ml. of 2 N sodium hydroxide in three portions at five-minute intervals such that the reaction mixture remained alkaline. The reaction mixture was magnetically stirred throughout the addition and for an additional 30 min. A white, sticky solid precipitated. It was filtered on a sintered glass

funnel and washed with two 10-ml. portions of cold water. The filtrate was acidified with concentrated hydrochloric acid (congo red) and cooled. The precipitate was identified as benzoic acid. The precipitate from the basic solution was stirred with 10 ml. of water. The white powder formed weighed 595 mg. (67%), m.p. 190-193° (corr.). After three recrystallizations from ethyl acetate the sample showed m.p. 193.5-194.5° (corr.).

Anal.	$C_{19}H_{18}N_2O_4$ (338.37)	Calc'd:	C, 67.43; H, 5.35; N, 8.28
	$C_{19}H_{16}N_2O_3$ (320.35)	Calc'd:	C, 71.24; H, 5.03; N, 8.75
		Found :	C, 70.05; H, 5.91; N, 8.61
			C, 70.20; H, 5.95; N, 8.69
			C, 70.08; H, 5.69; N, 8.54

The n.m.r. spectrum of the derivative in trifluoroacetic acid solution (TMS) (c, 20%) showed the following absorptions: a complex peak centered at 2.27 τ (10H) and three other broad peaks at 6.12 τ (1.94H), 6.78 τ (1.92H), and 7.69 τ (1.98H).

Flavianate Salt Derivative

To a solution of 50 mg. (0.28 mmole) of the crude cis-3-aminopyrrolidine-2-carboxylic acid sulfate in 3 ml. of water was added 2 ml. of a 25% solution of 2,4-dinitro-1-naphthol-7-sulfonic acid (flavianic acid). A yellow precipitate formed within a few minutes. The mixture was heated to boiling and 15 ml. of water was added. The hot solution was centrifuged and the supernatant solution was heated to boiling. After cooling, the yellow needles were collected and weighed 95 mg. (40%). An analytical sample was prepared by recrystallizing the product three more times from water, m.p. 229-231° dec. (corr.).

Anal. $C_{30}H_{28}N_8S_2O_{18}$	Calc'd:	C, 42.24; H, 3.31; N, 13.14; S, 7.52
(852.45)	Found :	C, 42.08; H, 4.01; N, 13.39; S, 8.36
		C, 43.52; H, 4.83; N, 12.75; S, 7.24
		C, 42.66; H, 4.15; N, 13.61; S, 7.90

4-Aminopyrrolidine-2,4-dicarboxylic Acid

Preparation and Properties

1,2-Dicarbethoxy-4-pyrrolidone was prepared by the method of Kuhn and Osswald (28). The preparation was distilled at 109-115°/0.5 mm [lit. (28) b.p. 170-175°/12 mm].

A solution of 20.0 g. (0.087 mole) of 1,2-dicarbethoxy-4-pyrrolidone, 11.3 g. (0.174 mole) of potassium cyanide, 9.3 g. (0.174 mole) of ammonium chloride, and 45.0 ml. of 30% aqueous ammonia in 800 ml. of methanol was allowed to stand at room temperature for six days. The methanol was evaporated *in vacuo* and the residue was dissolved in 40 ml. of water saturated with sodium chloride. The suspension was extracted with four 150-ml. portions of chloroform and the chloroform extract was dried for a few minutes over magnesium sulfate. The dry chloroform extract was extracted with two 30-ml. portions of 1.2 N hydrochloric acid. The acidic solution was made basic with potassium carbonate, saturated with sodium chloride and extracted with four 150-ml. portions of chloroform. Both chloroform extracts were dried over magnesium sulfate and evaporated to dryness *in vacuo*. The neutral fraction weighed 2.50 g. and the basic fraction weighed 8.80 g. (40% crude yield). The infrared spectrum of the basic fraction showed an absorption at 4.45 μ. Reactions were run for periods of 2, 6, 9, 13, and 16 days. The basic fraction from the six-day reaction had the largest weight and showed the most intense

4.45 μ band.

A solution of 3.4 g. of the basic fraction in 75 ml. of 6 N hydrochloric acid was boiled under reflux for 70 hours. The solution was evaporated to dryness *in vacuo* and neutralized with IR-45(OH^-) in absolute ethanol. Ethanol was evaporated, *in vacuo*, five times from this mixture to remove ammonia. The last ethanol mixture showed a negative test with Nessler's reagent. The amino acid was washed from the resin with water and finally with 1 N hydrochloric acid until the washing solution was ninhydrin negative. The acidic solution was neutralized with IR-45(OH^-) to pH 2.5, combined with the neutral solution and evaporated to about 30 ml. *in vacuo*. The resulting brown solution was decolorized with charcoal. The suspension was filtered and the filtrate was evaporated to dryness *in vacuo* and gave 1.99 g. of material. Crystallization from water-ethanol afforded 1.74 g. of white needles (30%, based on starting ketone). An analytical sample was prepared by crystallizing the solid three more times from water-ethanol. On heating the amino acid changed to a dark color at about 300° but did not melt up to 350°.

Anal. $C_6H_{10}O_4N_2$ (174.16) Calc'd: C, 41.38; H, 5.79; N, 16.08
Found : C, 41.13; H, 6.25; N, 15.88

The amino acid had R_F values 0.78, and 0.26 in water and BAW, respectively, and gave a purple-brown color with the ninhydrin reagent. The infrared spectrum of the compound as a potassium bromide pellet is given as Fig. 5. The n.m.r. spectrum in deuterium oxide and potassium carbonate (DSS) (*c*, 20%) showed complex absorptions from 5.56 to 6.13 τ (1H), 6.15 to 6.76 τ (1.62H), and 7.00 to 8.30 τ (1.67H). This spectrum

is given as figure 6.

2,4-Dinitrophenyl Derivative

The 2,4-dinitrophenyl derivative was prepared according to the general method used for preparing 2,4-dinitrophenylamino acids (25). To a solution of 231 mg. (1.32 mmole) of 4-aminopyrrolidine-2,4-dicarboxylic acid in 20 ml. of water at room temperature was added 1.0 g. (11.9 mmole) of sodium bicarbonate. To the solution was added a solution of 1.0 g. (5.35 mmole) of 2,4-dinitrofluorobenzene in 20 ml. of ethanol. The reaction mixture was allowed to stand at room temperature with occasional swirling for four hours. The ethanol was evaporated *in vacuo* and the mixture was acidified with hydrochloric acid and cooled in the refrigerator. The yellow solid was filtered on a sintered glass funnel, washed with two 10-ml. portions of water and then with three 15-ml. portions of benzene. The air-dried yellow powder weighed 525 mg. (78%), m.p. 245-250° dec. (corr.). Three recrystallizations from water gave an analytical sample, m.p. 247-250° dec. (corr.).

Anal. $C_{18}H_{14}N_6O_{12}$ (506.36) Calc'd: C, 42.70; H, 2.79; N, 16.60
Found : C, 42.70; H, 3.13; N, 16.57

Flavianate Salt Derivative

To a solution of 100 mg. (0.57 mmole) of 4-aminopyrrolidine-2,4-dicarboxylic acid in 10 ml. of water was added 5.0 ml. of a 25% solution of 2,4-dinitro-1-naphthol-7-sulfonic acid (flavianic acid). The reaction mixture was allowed to stand at room temperature for 30 min. and the solution was evaporated *in vacuo* to about 5.0 ml. The reaction mixture was heated to boiling and enough water was added to dissolve any undissolved solid. The yellow needles that formed on cooling were

collected and weighed 295 mg. (64%), m.p. 245-249° dec. (corr.). An analytical sample was prepared by recrystallizing the product three times from water, m.p. 250-253° dec. (corr.).

Anal.	$C_{26}H_{22}N_6S_2O_{20}$ (802.64)	Calc'd:	C, 38.90; H, 2.76; N, 10.47; S, 8.00
		Found :	C, 38.90; H, 3.81; N, 10.45; S, 7.85

Attempted Preparation of 4-Guanidopyrrolidine-2,4-dicarboxylic Acid

Aminoguanidine nitrate was prepared from aminoguanidine bicarbonate (Eastman 1932). To a suspension of 5.0 g. (37.0 mmole) of aminoguanidine bicarbonate in 20 ml. of absolute ethanol 8 ml. of concentrated nitric acid was added dropwise. When the carbon dioxide evolution had stopped, the mixture was cooled and the precipitate was collected on a sintered glass funnel. The white solid was washed with three 5-ml. portions of cold absolute ethanol and recrystallized from hot absolute ethanol. The white needles obtained weighed 3.0 g. (60%), m.p. 144-145° (corr.), [lit. (29), m.p. 144°].

1-Guanyl-3,5-dimethylpyrazole nitrate was prepared from acetylacetone and aminoguanidine nitrate according to the method of Thiele and Dralle (30). The product obtained had m.p. 167-168° (corr.) [lit. (30), m.p. 168°).

To 1750 ml. of 1 N sodium hydroxide solution was added 34.0 g. (170 mmole) of 1-guanyl-3,5-dimethylpyrazole nitrate. The free base was extracted at once with three 150-ml. portions of ethyl acetate and the combined extracts were dried over magnesium sulfate. The supernatant liquid was evaporated to dryness in vacuo. The resulting oil was dissolved in 45 ml. of water. The aqueous solution was mixed with 8.80 g.

of the crude basic fraction, obtained from the strecker reaction using 1,2-dicarbethoxy-4-pyrrolidone, and 10 ml. of triethylamine. The reaction mixture was heated at 110° under reflux for three hours. Papergram analysis of the product in BAW is shown below.

Compound	R_F (BAW)	Weber	Ninhydrin
1-Guanyl-3,5-dimethyl-pyrazole Nitrate	0.70	Blue	----
Product	0.58	Pink	----
	0.70	Blue	----

The solution was evaporated to dryness *in vacuo*. The residue was dissolved in 100 ml. of 6 N hydrochloric acid and the solution was boiled under reflux for 55 hr. The reaction mixture was evaporated *in vacuo* to about 50 ml. and treated batchwise with IR-45(OH^-) resin until the pH rose to 3.0. The supernatant liquid was passed over an IR-400(OH^-) column containing 900 ml. of resin. The resin was washed with 2 l. of water and the eluate evaporated *in vacuo* to about 50 ml. This aqueous solution was extracted with four 80-ml. portions of ethyl acetate. Papergram analysis of both the ethyl acetate fraction and the aqueous fraction are shown below. The aqueous layer was evaporated to dryness *in vacuo*. The tan solid obtained weighed 4.80 g. The solid was dissolved in 10 ml. of water and pipetted onto a carbon column containing 150 g. of 1:2 acid-washed celite:Darco G-60 that had been pretreated with three per cent oleic acid. The column was eluted with water (810 fractions of about

20 ml. each). Papergrams on every other fraction were developed in BAW and sprayed with the Weber reagent. Fractions 1-40 showed no positive spots. Fractions 41-85 revealed a pink spot in BAW with R_F 73. Frac-

Compounds	R_F (BAW)	Weber	Ninhydrin
1-Guanyl-3,5-dimethyl-pyrazole Nitrate	0.70	Blue	----
Ethyl Acetate Fraction	0.70	Blue	----
Aqueous Fraction	0.66	Pink	----

tions 86-810 showed a faint pink spot of the same R_F. Fractions 41-85 were combined and evaporated <u>in vacuo</u> to about 30 ml. The suspension was filtered hot on a sintered glass funnel and the filtrate was evaporated to dryness <u>in vacuo</u>. The almost colorless residue weighed 3.080 g. Fractions 86-810 were combined and treated in the same way. This gave 0.570 g. of tan residue. The product from the main fraction was dissolved in 20 ml. of hot water and centrifuged. To the supernatant liquid was added hot absolute ethanol until a cloudiness appeared. The mixture was cooled and filtered. The process was repeated three more times to get rid of the column throw. The aqueous solution was evaporated to dryness <u>in vacuo</u>. The residue was recrystallized from methanol-ethyl acetate and gave 695 mg. of light tan needles, m.p. 226-228° dec. (corr.). An analytical sample was prepared by recrystallizing the product three more times from methanol-ethyl acetate, m.p. 230-231° dec. (corr.).

Anal.	$C_7H_{12}N_4O_4$ (216.20)	Calc'd:	C, 38.89; H, 5.59; N, 25.92
	$C_3H_9N_3O_2$ (119.13)	Calc'd:	C, 30.25; H, 7.62; N, 35.27
		Found :	C, 30.58; H, 7.50; N, 34.76
			C, 30.09; H, 7.48; N, 35.59

The compound gave a negative Sakaguchi test. The infrared spectrum as a potassium bromide pellet, showed, among others, absorptions at 2.94, 3.22, 6.02, 6.46, and 7.11 μ. The n.m.r. spectrum in deuterium oxide (DSS) (c, 10%) showed only a sharp singlet at 7.97 τ. The n.m.r. spectrum in trifluoroacetic acid (TMS) (c, 15%) showed two absorption peaks, a broad one at 3.86 τ and a sharp singlet at 7.81 τ. The ratio of the areas were 5 to 2, respectively.

The n.m.r. spectrum of an authentic sample of guanidine acetate as a potassium bromide pellet was identical with that of the unknown. The n.m.r. spectrum of the guanidine acetate in deuterium oxide (DSS) (c, 15%) showed a sharp singlet at 8.06 τ. A papergram analysis of the unknown and guanidine acetate is shown below.

Compound	R_F (BAW)	R_F (PW)	R_F (PAW)	Weber
Unknown	0.60	0.58	0.66	Pink
Guanidine Acetate	0.60	0.60	0.66	Pink

2,4-Dinitrophenylviomycidine

The method used for the preparation of 2,4-dinitrophenylviomycidine was an adaption of a general method for the preparation of 2,4-dinitrophenylamino acids (25). To a solution of 202 mg. (0.98 mmole) of viomycidine

hydrochloride in 15 ml. of water was added 1.0 g. of sodium bicarbonate. To this solution was added a solution of 1.10 g. (6.0 mmole) of 2,4-dinitrofluorobenzene in 20 ml. of ethanol. The suspension was stirred magnetically for two hours at room temperature. The ethanol was evaporated in vacuo and the residue was acidified with hydrochloric acid. The orange solid was collected on a sintered glass funnel and washed well with water, benzene, and chloroform. The air-dried solid weighed 323 mg. (88%) and had m.p. 171-172°. An analytical sample was prepared by recrystallizing the compound three more times from water. It showed m.p. 172-173° (corr.). The analytical sample was dried to constant weight.

Anal. $C_{12}H_{12}O_6N_6 \cdot 2H_2O$ (372.20) Calc'd: C, 38.71; H, 4.33; N, 22.58
Found : C, 38.79; H, 4.49; N, 22.57

Ozonolysis of 2,4-Dinitrophenylviomycidine

Ozone generated by a Welsbach Model T-23 ozone generator was bubbled through a solution of 216 mg. (0.58 mmole) of 2,4-dinitrophenylviomycidine in 50 ml. of 80% formic acid for one hour at 0°. One milliliter of 30% hydrogen peroxide was added and the reaction mixture was allowed to stand at room temperature overnight. The solution was evaporated to dryness in vacuo. The yellow solid obtained weighed 200 mg. TLC in BMA indicated two yellow spots, a light yellow one of R_F 0.52 and a deep yellow one of R_F 0.75. 2,4-Dinitrophenylviomycidine and 2,4-dinitroaniline had R_F values of 0.50 and 0.75, respectively, in BMA. A TLC plate in BMA was sprayed with the Weber reagent and gave one light blue spot of R_F 0.56 (guanidine showed a pink spot of R_F 0.48). No ninhydrin positive spots were observed. The yellow residue was dissolved in 20 ml. of

concentrated hydrochloric acid and hydrolyzed in a pressure bottle for 6 hrs. on a steam bath. One hundred milliliters of t-butyl alcohol was added and the solution was evaporated to dryness in vacuo. Papergram and TLC analyses of the yellow residue are shown in Tables 6 and 7, respectively.

Table 6. Papergram Analysis of the Ozonolysis Product of 2,4-Dinitrophenylviomycidine.

Compound	R_F (BAW)	R_F (PW)	Color	Weber	Ninhydrin
2,4-Dinitroaniline	0.69	0.81	Yellow	----	----
Guanidine	0.45	0.38	----	Pink	----
DNP-Viomycidine	0.65	0.64	Yellow	Pink	----
Product	0.68	0.81	Yellow	----	----
	0.45	0.38	----	Pink	----
	0.38	0.26	----	Pink	----

Table 7. TLC Analysis of the Ozonolysis Product of 2,4-Dinitrophenylviomycidine.

Compound	R_F (BMA)	Color	Weber
Guanidine	0.48	----	Pink
2,4-Dinitroaniline	0.78	Yellow	----
DNP-Viomycidine	0.50	Yellow	Pink
DNP-Aspartic Acid	0.24	Yellow	----
Product	0.78	Yellow	____
	0.49	----	Pink
	0.06	----	Pink

Ozonolysis of 2,4-Dinitrophenylaspartic Acid

2,4-Dinitrophenylaspartic acid was prepared by the general method used for the preparation of 2,4-dinitrophenylamino acids (25). The

product had m.p. 186-187° (corr.) [lit. (31), m.p. 186-187°].

A solution of 200 mg. (0.67 mmole) of 2,4-dinitrophenylaspartic acid in 50 ml. of 80% formic acid was ozonized under the same conditions used for the ozonolysis of 2,4-dinitrophenylviomycidine. The product, after ozonolysis and acid hydrolysis, weighed 190 mg. TLC analyses of this yellow product in BMA indicated two yellow spots: a main spot R_F 0.79 and a very light spot R_F 0.25. Table 7 gives reference R_F values of known substances.

Reaction of 2,4-Dinitrophenylaspartic Acid with Acidic Potassium Permanganate Solution

To 45 ml. of 0.01 N sulfuric acid was added 48 mg. (0.30 mmole) of potassium permanganate. To this solution was added 90 mg. (0.30 mmole) of 2,4-dinitrophenylaspartic acid. The reaction mixture was heated under reflux on the steam bath. The permanganate solution became colorless after 5 min. The reaction mixture was cooled and the yellow precipitate that had formed was collected on a sintered glass funnel. The air-dried solid weighed 42 mg. (76%) and had m.p. 177-179° (corr.). TLC analyses in BMA indicated the same R_F value as 2,4-dinitroaniline. No depression in melting point was observed when a mixture melting point with an authentic sample of 2,4-dinitroaniline was determined.

Ozonolysis of Acetylviomycidine

Acidic Preparation

Acetylviomycidine was prepared using the method of Miller (11). A solution of 58 mg. (0.28 mmole) of viomycidine hydrochloride in 2.0 ml. of water was cooled to 10° and enough absolute ethanol was added to bring

cloudiness. Two milliliters of acetic anhydride was added and the reaction mixture was kept in the refrigerator for 66 hr. The solution was evaporated to dryness *in vacuo*. The deep tan residue was dissolved in 50 ml. of 80% formic acid and ozonized at 0° for 30 min. One milliliter of 30% hydrogen peroxide was added and the reaction mixture was allowed to stand at room temperature overnight. The colorless solution was evaporated to dryness *in vacuo*. Ten milliliters of 6 N hydrochloric acid was added and the reaction mixture was heated on a steam bath under reflux for 16 hr. Papergram analyses of the product and certain other compounds are shown below. The colors of the spots with R_F values 0.47 and 0.52 had

Compound	R_F (BAW)	Ninhydrin	Weber
Aspartic Acid	0.47	Purple	----
Viomycidine Hydrochloride	0.34	Purple	Pink
Guanidine	0.52	------	Pink
Product	0.18	Blue	----
	0.34	Purple	Pink
	0.47	Purple	----
	0.52	------	Pink

very low intensity compared with the others.

Basic Preparation

A mixture of 264 mg. (1.30 mmole) of viomycidine hydrochloride, 30 ml. of acetic anhydride, and 30 ml. of dry pyridine was allowed to stand at room temperature for 24 hr. The suspension was heated on a steam bath for 8 hr. and filtered. The residue was reacetylated in the same way. Filtration left only a very small residue. The filtrates from both runs were combined, diluted with 60 ml. of water, and allowed

to stand at room temperature for 2 hr. The reddish-brown solution was evaporated to dryness *in vacuo*. The brown residue was dissolved in 100 ml. of 80% formic acid and ozone was bubbled through the solution for one hour at 0°. Two milliliters of 30% hydrogen peroxide was added and the mixture was allowed to stand at room temperature overnight. The colorless solution was evaporated to dryness *in vacuo*, mixed with 30 ml. of 6 N hydrochloric acid, and the solution was heated on a steam bath under reflux for 18 hr. The dark solution was evaporated to about 10 ml. *in vacuo*. Papergram analysis of the black solution is shown below.

Compound	R_F (BAW)	R_F (Isobutyric Acid)	R_F (PAW)	Ninhydrin	Weber
Viomycidine	0.34	0.16	0.66	Purple	Pink
Guanidine	0.52	0.45	0.72	------	Pink
Aspartic Acid	0.47	0.22	0.70	Purple	----
β-Alanine	0.54	----	0.80	Purple	----
Product	0.34	0.16	0.66	Purple	Pink
	0.47	0.23	0.71	Purple	----
	0.52	0.46	0.73	------	Pink

The color of the spot corresponding to viomycidine had very low intensity compared with those corresponding to aspartic acid and guanidine.

The black mixture from the basic preparation was neutralized with sodium hydroxide solution to pH 2 and then neutralized with sodium bicarbonate solution. To this solution was added 2.0 g. of sodium bicarbonate and a solution of 2.0 g. (10.7 mmole) of 2,4-dinitrofluorobenzene in 30 ml. of ethanol. The reaction mixture was allowed to stand at room temperature for 3 hrs. Ethanol was evaporated *in vacuo* and the suspension was acidified with hydrochloric acid. The reaction mixture was cooled in

the refrigerator for several hours and then extracted with five 50-ml. portions of ethyl acetate. The ethyl acetate extract was dried over magnesium sulfate and evaporated *in vacuo*. The yellow-red oil was dissolved in 10 ml. of acetone and analyzed by TLC. The results are shown immediately below. The solution was evaporated to dryness *in vacuo*.

Compound	R_F (BA)	R_F (BMA)	Color
DNP-Viomycidine	0.42	0.46	Yellow
DNP-Aspartic Acid	0.24	0.32	Yellow
2,4-Dinitrophenol	0.74	0.68	Yellow
Product	0.00	0.00	Brown
	0.24	0.34	Yellow
	0.76	0.70	Yellow

The viscous oil obtained weighed 590 mg. Crystallization attempts were not successful. The oil was dissolved in 8 ml. of ethyl acetate and applied to a silicic acid column (3.5 cm. diameter, 18 cm. high). The column was eluted with ethyl acetate (15 fractions of about 6 ml. each). A yellow band was eluted. TLC analyses of all fractions indicated the same compounds previously shown, with the exception of the R_F 0.00 spot. All the yellow fractions were combined and evaporated *in vacuo*. The yellow-brown viscous residue weighed 505 mg. The residue was redissolved in 5 ml. of ethyl acetate and applied to 1 mm thick silica gel G thin layer plates with a spot of 2,4-dinitrophenylaspartic acid as a reference on each plate. A total of 12 plates having 20 cm. width were used. The plates were developed in BA. A total of four bands were observed that had R_F values of 0.20 (yellow), 0.30 (brown), 0.52 yellow), and 0.78 (yellow). 2,4-Dinitrophenylaspartic acid had R_F 0.20 (yellow).

The yellow band that corresponded to 2,4-dinitrophenylaspartic acid was removed and the compound was extracted from the adsorbent with 5% formic acid in acetone. The yellow solution was evaporated to dryness *in vacuo* and gave 165 mg. of brown-yellow solid. The residue was analyzed by TLC and the results are shown below. All attempts to crystallize the preparation failed.

Solvent Systems	Product	DNP-Aspartic
BA	0.21	0.21
BMA	0.33	0.32
MA	0.72	0.74
BAW	0.46	0.47
PAW	0.30	0.32
Ethyl Acetate	0.00	0.00
n-Butanol	0.00	0.00
Methanol	0.06	0.06
Ethanol	0.10	0.08

Another 100 mg. (0.49 mmole) of crude viomycidine hydrochloride was mixed with 12. ml. of dry pyridine and 12 ml. of acetic anhydride. The acetylation, ozonization, and acid hydrolysis of the ozonized product were carried out in the same way described above. The dark solution obtained from the hydrolysis was evaporated to about 1 ml. *in vacuo*. The black solution was applied to three 12-cm. wide strips of Whatman No. 17 chromatography paper. The papergrams were developed in BAW. A 0.5 cm. wide section was cut from each strip and sprayed with ninhydrin. The sections corresponding to the high R_F spot (0.47) were cut and sewed together in long strips connected with Whatman No. 1 paper. The long bridged sections were eluted with water by descending chromatography.

The combined eluates were evaporated *in vacuo* and gave 150 mg. of a partially crystalline tan solid. The solid was dissolved in 3 ml. of water. The solution was pipetted onto a carbon column 1.0 cm. in diameter and containing 10 g. of 1:2 acid-washed celite 545:Darco G-60 that had been pretreated with three per cent oleic acid. The packed column was 27.0 cm. high. The column was eluted with water (15 fractions of about 4 ml. each). A drop was drawn from each fraction and tested with ninhydrin reagent. Fractions 1-6 were negative, fractions 7-11 indicated a strong positive test, and fractions 12-15 indicated a very weak test. Fractions 7-11 were combined and lyopholized. The white compound obtained weighed 115 mg. The compound was dissolved in 1 ml. of hot distilled water, centrifuged, and the supernatant liquid was allowed to cool slowly to room temperature. The solution was then kept in the refrigerator overnight. The white crystalline solid obtained was collected on a Büchner funnel. The dry crystals weighed 29 mg. The filtrate was concentrated to 0.4 ml. The solid obtained upon cooling was collected and weighed 7 mg. The combined crystalline product weighed 36 mg. (53%, based on viomycidine hydrochloride).

A sample of the first crop of the unknown showed $[\alpha]_D^{27^\circ} = 0.0^\circ$ (*c*, 7.68, 1 *N* hydrochloric acid). An authentic sample of *L*-aspartic acid showed $[\alpha]_D^{27^\circ} = +22.86^\circ$ (*c*, 8.00, 1 *N* hydrochloric acid). The infrared spectrum of the unknown and that of an authentic sample of *DL*-aspartic acid taken as a potassium bromide pellet were superimposable. The n.m.r. spectrum of the unknown in 0.5 *N* hydrochloric acid solution (TMS, external standard) (5%) was identical to the n.m.r. spectrum of a similar solution of aspartic acid.

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photometrically according to a standard procedure (32). A 10 ml. aliquot of the hydrolysate was mixed with 5 ml. of clear Nessler's reagent in a 50 ml. volumetric flask. The flask was filled to the mark with distilled water. The solution was compared photometrically with a standard solution of ammonium chloride treated with Nessler's reagent in the same proportion at the same time. A Spectronic 20 Bausch and Lomb spectrometer was used in the analysis. The amount of ammonia liberated in the hydrolysis was 4.55 mg. (0.268 mmole, 3.7%).

Barium Hydroxide Hydrolysis of Viomycin Hydrochloride

A three-necked 1 l. round-bottomed flask was fitted with a 500 ml. dropping funnel and two one-holed rubber stoppers, in each of which was inserted a glass tube. The funnel was filled with distilled water, which was added as needed to keep the volume of the basic solution constant. Nitrogen that had been bubbled through concentrated sulfuric acid was admitted through one tube, which was inserted to the bottom of the flask, to force any volatile base formed out the other tube. The other tube dipped into a solution of 10.0 g. of boric acid in 250 ml. of distilled water. The solution was back titrated at intervals with 0.0332 N hydrochloric acid to a screened methyl red end point. The indicator was prepared by mixing solutions of 0.26 g. of methyl red in 100 ml. of ethanol and 0.186 g. of methylene blue in 100 ml. of ethanol. To 5.005 g. (6.30 mmole) of viomycin hydrochloride (assumed mol. wt. 798) was added 600 ml. of aqueous barium hydroxide solution (saturated at room temperature). The mixture was heated on a steam bath. The rate of evolution of volatile base (ammonia) is given in Table 8 and Figure 7.

Table 8. Moles of Ammonia Liberated from the Base Hydrolysis of Viomycin and Some Related Compounds.

Time Hours	Moles of Volatile Base per Mole of Compound: Viomycin Hydrochloride	Urea	Viomycidine(9)	L-Serine	α,β-Diaminopropionic Acid
0:20	0.05	----	----	----	----
1:15	0.17	----	----	----	----
2:00	----	----	0.18	----	----
2:30	0.64	----	----	----	----
3:30	0.86	----	----	----	----
4:00	----	0.12	0.53	----	----
5:00	1.14	0.20	----	----	----
6:00	----	----	0.90	----	----
8:00	----	0.40	1.13	----	----
8:15	1.44	----	----	----	----
9:30	----	0.49	----	----	----
10:00	----	----	1.53	----	----
10:30	----	0.56	----	----	----
11:15	1.67	----	----	----	----
21:00	----	----	1.94	----	----
22:00	----	1.00	----	----	----
22:30	2.15	----	----	----	----
24:00	----	1.11	2.08	----	----
25:00	----	----	----	0.02	----
26:30	2.40	----	----	----	----
27:00	----	1.22	2.20	----	----
30:00	----	1.32	----	----	----
30:30	2.54	----	----	----	----
31:00	----	----	2.32	----	----
33:00	----	1.41	----	----	----
34:30	----	1.45	----	----	----
35:00	2.64	----	----	----	----
36:00	----	----	2.47	0.03	----
42:00	----	----	----	----	0.01
46:00	2.88	1.77	----	----	----
48:00	----	----	----	0.05	----
48:30	----	1.81	2.58	----	----
51:00	2.98	----	----	----	0.016
54:00	----	1.91	----	----	----
55:00	3.04	----	----	----	----
70:00	3.24	----	----	0.07	----
74:00	----	2.04	----	----	----
77:00	----	----	2.75	----	----
79:30	----	----	----	0.08	----
88:00	----	2.07	----	0.095	0.02

After heating on the steam bath for 70 hr., the above viomycin hydrolysate was acidified to pH 1 with sulfuric acid. The barium sulfate precipitate was filtered by gravity and washed with two 100-ml. portions of water. The filtrate was treated batchwise with IR-45(OH^-) ion exchange resin to pH 5.0. The supernatant liquid was passed over an IR-45(Cl^-) ion exchange column containing 300 ml. of resin. The column was washed with 600 ml. of water. The eluate was evaporated to about 20 ml. in vacuo and lyopholized. This gave 2.186 g. of tan solid. Two dimensional papergrams indicated that four main ninhydrin-positive compounds were present. The R_F values obtained were (BAW/PW) 0.58/0.51, 0.44/0.32, 0.37/0.30, and 0.24/0.10. The R_F values for alanine, serine, β-lysine, and diaminopropionic acid were 0.58/0.52, 0.45/0.32, 0.38/0.29, and 0.23/0.09, respectively. The IR-45(OH^-) ion exchange resin used in neutralizing the above hydrolysis was stirred with hydrochloric acid solution to a pH 1. The supernatant liquid was evaporated to about 20 ml. in vacuo, decolorized with activated charcoal and evaporated to dryness in vacuo. This gave 320 mg. of a brown gum. A papergram in PW showed that four main ninhydrin-positive compounds were present. The R_F values were 0.50, 0.32, 0.29, and 0.10. This residue gave a negative aniline blue test for oxalic acid (18).

The main hydrolysate (2.186 g.) was passed over an IRC-50(H^+) ion exchange column containing 300 ml. of resin. The column was washed with 600 ml. of distilled water. The eluate was evaporated to about 20 ml. in vacuo and lyopholized. This gave 1.858 g. of a tan glassy solid. A papergram in PW indicated all the spots shown before. The IRC-50(H^+) column was washed with 1 N hydrochloric acid until the eluate was ninhydrin

negative. The eluate was evaporated *in vacuo* and gave 280 mg. of a brown solid. A papergram in PW indicated a main ninhydrin-positive compound corresponding to α,β-diaminopropionic acid and a minor compound corresponding to β-lysine. The neutral fraction (1.858 g.) was dissolved in 18 ml. of water and applied to thick chromatography paper (Whatman No. 17) in the ratio of 35 mg. per 12 cm. wide strip. The chromatograms were developed in PW. a 0.5 cm. wide section was cut from each strip and sprayed with ninhydrin. All the sections corresponding to the high R_F spot (0.50) were cut and sewed together in long strips connected with Whatman No. 1 paper. The long bridged sections were eluted with water by descending chromatography. The combined eluates were evaporated *in vacuo* to about 15 ml. and lyopholized. This gave 410 mg. of a light tan solid. The R_F value in PW of the preparation was 0.52.

The above solid was dissolved in 5 ml. of water. This solution was pipetted onto a column 2.0 cm. in diameter containing 45 g. of 1:2 acid-washed celite 545:Darco G-60 that had been pretreated with three per cent oleic acid. The packed column was 50 cm. high. The column was eluted with water (30 fractions of about 16 ml. each). Papergrams on all fractions were developed in BAW and sprayed with ninhydrin reagent. Fractions 1-17 showed no ninhydrin-positive spots; fractions 18-23 revealed a deep purple spot with the same R_F as alanine, and fractions 24-30 showed a faint purple spot of the same R_F. Fractions 18-23 were combined and evaporated *in vacuo* to about 15 ml. and lyopholized. The white solid obtained weighed 238 mg. Fractions 24-30 gave 32 mg. of a light brown solid.

A sample of the main fraction of the unknown showed $[\alpha]_D^{25^\circ} = 0.0^\circ$ (*c*, 9.80, 5.0 *N* hydrochloric acid). The n.m.r. spectrum of the unknown in

deuterium oxide (TMS, external standard) (c, 10%) showed peaks at 6.22 τ (1H, quartet, J = 7.5 cps), and 8.56 τ (3.1H, doublet, J = 7.5 cps). The n.m.r. spectrum of an authentic sample of DL-alanine in deuterium oxide (DSS) (c, 15%) showed peaks at 6.22 τ (1H, quartet, J = 7.5 cps) and 8.55 τ (3H, doublet, J = 7.5 cps).

The 2,4-dinitrophenyl derivative of the unknown was prepared according to a general method for the preparation of 2,4-dinitrophenylamino acids (25). To a solution of 130 mg. (1.45 mmole) of the main fraction of the unknown in 10 ml. of water was added 0.50 g. (5.95 mmole) of sodium bicarbonate. To this solution was added 0.50 g. (2.67 mmole) of 2,4-dinitrofluorobenzene in 10 ml. of ethanol. The yellow suspension was allowed to stand at room temperature for two hours with occasional swirling. The ethanol was evaporated in vacuo and the mixture acidified with hydrochloric acid. A yellow sticky precipitate formed. The reaction mixture was kept in the refrigerator overnight and extracted with four 20-ml. portions of ether. The ether extracts were dried over magnesium sulfate and evaporated in vacuo. This gave a yellow gum. This residue was mixed with 15 ml. of water and extracted with three 20-ml. portions of benzene to remove 2,4-dinitrophenol. The aqueous layer was evaporated in vacuo at about 40°. The yellow residue was recrystallized from water and gave 12 mg. of yellow crystals, m.p. 155-160° (corr.). The 2,4-dinitrophenyl derivative of an authentic sample of DL-alanine prepared by the same method showed m.p. 173-174° (corr.). The infrared spectrum of the derivative and that of the authentic sample, as potassium bromide pellets, were identical.

Another sample of viomycin hydrochloride (1.240 g., 1.55 mmole)

was hydrolyzed in 100 ml. of saturated barium hydroxide solution on a steam bath for 73 hr. The reaction mixture was cooled to room temperature and filtered by gravity. The filtrate was passed over an IRC-50(H^+) ion exchange column containing 260 ml. of resin. The column was washed with 600 ml. of water and the eluate was evaporated to dryness *in vacuo*. The tan solid obtained weighed 686 mg. The solid was stirred with two 80-ml. portions of hot absolute ethanol and the ethanol solution was evaporated to dryness *in vacuo*. The solid residue obtained was heated under reflux with 50 ml. of benzene for one hour. The benzene suspension was centrifuged and concentrated to about 5 ml. The solution was cooled and the resulting light tan solid was collected. After drying in air it weighed 4 mg., m.p. 144-150° (dec.). A papergram on the unknown, pyrrole-2-carboxylic acid, and pyrrole-3-carboxylic acid was run in *n*-BAW. The chromatogram was sprayed with Erhlich's reagent. The unknown and pyrrole-2-carboxylic acid showed one purple spot each, R_F 0.74; pyrrole-3-carboxylic acid revealed one blue spot, R_F 0.70.

The infrared spectra of pyrrole-2-carboxylic acid and the unknown as potassium bromide pellets were identical. The ultraviolet spectrum of the unknown compound in 95 per cent ethanol solution showed λ_{max} 262 mμ, ε, 10,000. The spectrum of pyrrole-2-carboxylic acid showed λ_{max} 261 mμ, ε, 11,000 (11).

A solution of 1.050 g. (1.24 mmole) of viomycin sulfate in 100 ml. of the saturated barium hydroxide solution was heated on a steam bath under reflux for 3 days. The reaction mixture was cooled to room temperature and acidified with sulfuric acid. To the suspension was added 100 ml. of water and about 100 ml. of solvent was distilled. The distillate

was titrated with 0.0255 N potassium hydroxide solution pH 7.42 (10.50 ml., or 0.251 meq. of base was needed). The neutral solution was evaporated to about 10 ml. in vacuo and lyopholized. The white solid obtained was dried further in a vacuum desiccator overnight and was found to weigh 25 mg. The n.m.r. spectrum of the solid in deuterium oxide (DSS) (c, 6%) showed one sharp peak 8.17 τ (singlet). An authentic sodium acetate solution in deuterium oxide (DSS) (c, 6%) showed one peak 8.13 τ (singlet).

A blank hydrolysis was carried out in the same way and the distillate was titrated with potassium hydroxide to pH 7.42. Only 0.0069 meq. of base was required.

Barium Hydroxide Hydrolysis of Urea

The hydrolysis of a solution of 521 mg. (8.70 mmole) of urea in 500 ml. of saturated barium hydroxide was carried out the same way as the hydrolysis of viomycin. The results are shown in Table 8 and Figure 7.

Barium Hydroxide Hydrolysis of α,β-Diaminopropionic Acid Hydrochloride

A solution of 1.023 g. (7.30 mmole) of α,β-diaminopropionic acid hydrochloride in 500 ml. of saturated barium hydroxide was hydrolyzed under the same condition adopted for the hydrolysis of viomycin. The results are shown in Table 8 and Figure 7.

Barium Hydroxide Hydrolysis of Serine

A solution of 1.020 g. (9.72 mmole) of serine in 500 ml. of saturated barium hydroxide was hydrolyzed the same way as that of viomycin.

The results are shown in Table 8 and Figure 7. After hydrolysis for four days the reaction mixture was acidified with sulfuric acid to pH 1. The barium sulfate was removed by filtration and the filtrate was treated batchwise with IR-45(OH^-) resin until the pH rose to 5.0. The supernatant liquid was evaporated to about 10 ml. *in vacuo*. A papergram analysis of this product is given below. The intensity of the low R_F spot was

Compound	R_F (BAW)	R_F (PW)	R_F (n-BAW)	Ninhydrin
Serine	0.54	0.23	0.06	Purple
Alanine	0.66	0.46	0.13	Purple
Unknown	0.54	0.23	0.06	Purple
	0.66	0.46	0.13	Purple

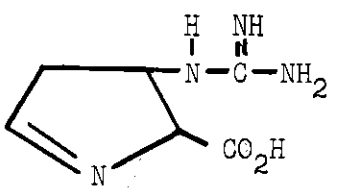
about five times that of the high R_F spot.

CHAPTER III

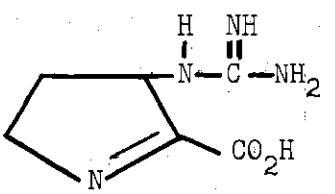
DISCUSSION OF RESULTS

Synthesis and Properties of Some Pyrrolidine Amino Acids

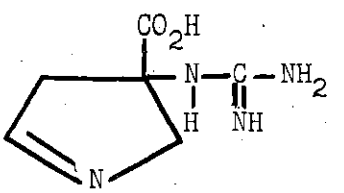
Previous workers in this laboratory (11,13) had proposed structures IV, V, and VIII for the structure of viomycidine, the guanido amino acid present in the acid hydrolysate of viomycin. There was some



IV



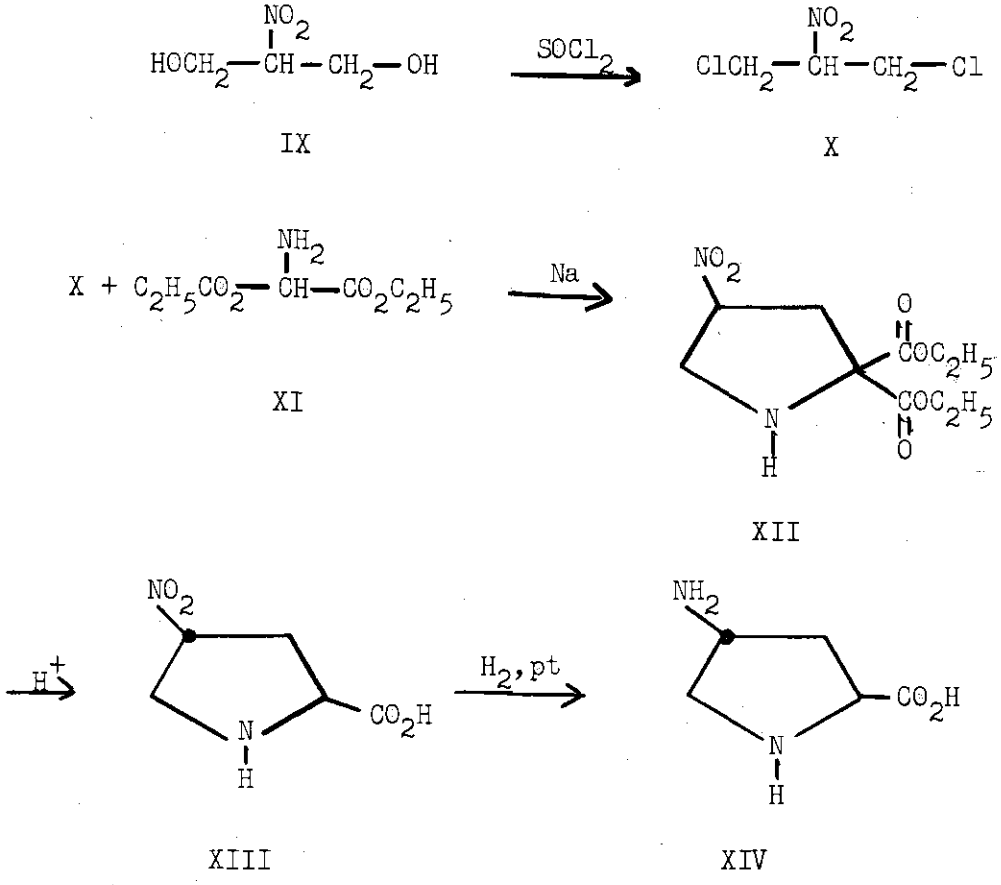
V



VIII

uncertainty about the positions of the functional groups, but it was considered certain that viomycidine had the Δ^1-pyrroline ring structure. It was also thought possible that hydrogenation of viomycidine under proper conditions, followed by barium hydroxide hydrolysis of the guanidine function to an amino group, would produce a pyrrolidine amino acid. With this in mind, the syntheses of several pyrrolidine amino acids were undertaken so that their properties could be studied and their identity or non-identity could be compared with those of dihydroviomycidine and those of the amino acid which would be obtained from the basic hydrolysis of the latter.

A proposed synthetic sequence for trans-4-aminopyrrolidine-2-carboxylic acid (XIV) is shown below. Decarboxylation of the malonic



IX X

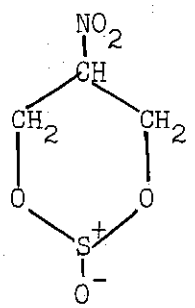
XI XII

XIII XIV

acid derivative resulting from XII would be expected to yield preponderantly the thermodynamically more stable trans isomer XIII.

2-Nitro-1,3-propanediol(IX) was prepared from formaldehyde and nitromethane, using the method of Den Otter (21). All attempts to prepare 2-nitro-1,3-dichloropropane(X) from 2-nitro-1,3-propanediol(IX) were unsuccessful. When a suspension of 2-nitro-1,3-propanediol in benzene was treated with thionyl chloride, a crystalline compound was readily obtained in 76 per cent yield. Elemental analysis failed to show the presence of chlorine; the data were consistent with the formula $C_3H_5NO_5S$ for the compound. The infrared spectrum showed important

bands at 6.40, 7.41, and 8.52 μ. Bellamy (33) states that the C-NO_2 group absorbs in the region 6.36 to 6.66 μ and 7.30 to 7.69 μ, and the dialkyl sulfite $S^{+}\!-\!O^{-}$ group absorbs in the region 8.23 to 8.70 μ. Thus, these results indicate that a condensation reaction of one mole of 2-nitro-1,3-propanediol(IX) and one mole of thionyl chloride had occurred to form the cyclic sulfite ester, 4-nitro-2,6-dioxatetrahydrothiapyran-1-oxide(XV). The nuclear magnetic resonance spectrum of XV in deuterio-

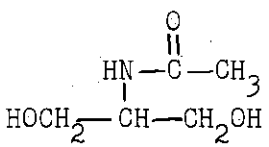


XV

acetone showed a complex series of absorptions between 4.50 and 5.40 τ. The spectrum was complicated because the protons of the methylene groups are not equivalent and the absorptions of the methylene protons are close to that of the methine proton. It is not known if the compound isolated is the cis or the trans isomer.

In another attempt to obtain a 2-substituted 1,3-dichloropropane, 2-nitro-1,3-propanediol(IX) was hydrogenated in 95 per cent ethanol, using platinum on carbon catalyst. The oily product, obtained in 99 per cent yield, could not be crystallized. The infrared spectrum of the crude product indicated that the nitro group had been completely reduced to the amino group. Attempts to replace the hydroxyl groups with chloride groups were also unsuccessful. The crude oil was then acetylated with cold

acetic anhydride in 50 per cent ethanol. This produced 2-acetamido-1,3-propanediol, XVI, in 40 per cent yield. The analytical data were consistent with formula $C_5H_{11}NO_3$ for the compound. The infrared spectrum of XVI



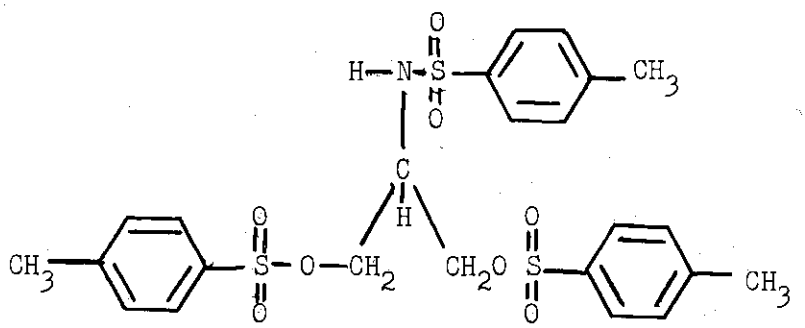
XVI

showed important absorptions at 2.90 (amide N-H and O-H), 6.15 (amide C=O), and 6.50 μ (amide N-H). The nuclear magnetic resonance spectrum in deuterium oxide showed a singlet at 7.98 τ (3H) for the acetyl hydrogens and a complicated series of peaks from 5.9 to 6.6 τ (5H) for the carbon-bound hydrogens. The bands are complicated because the protons of the methylene groups are nonequivalent and the absorptions are close together.

Attempts to replace the hydroxyl groups of XVI by chlorine atoms failed to give any identifiable chlorine-containing compound. When the acetamido compound (XVI) was reacted with p-toluenesulfonyl chloride in pyridine in an effort to prepare the 1,3-bis-tosyl derivative, a brown oil was obtained which could not be purified. The infrared spectrum of the crude product indicated, unexpectedly, a mixture of acetamido and acetyl carbonyl groups.

The crude 2-amino-1,3-propanediol was next reacted with p-toluenesulfonyl chloride in pyridine. The infrared spectrum of the crystalline product, obtained in 49 per cent yield, showed typical sulfonamide and sulfonate ester bands at 7.40 and 8.60 μ, respectively. Analytical data were

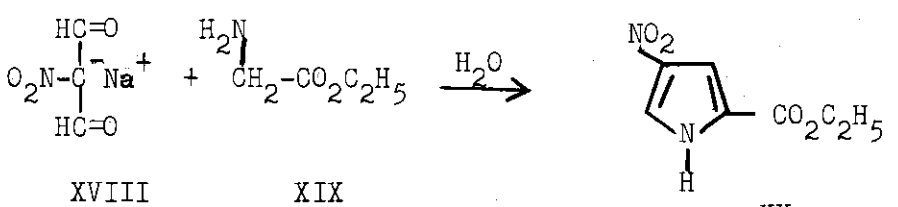
consistent with the formula $C_{24}H_{27}NO_8S_3$ and the product was assigned the structure tri-p-toluenesulfonyl-2-amino-1,3-propanediol (XVII).



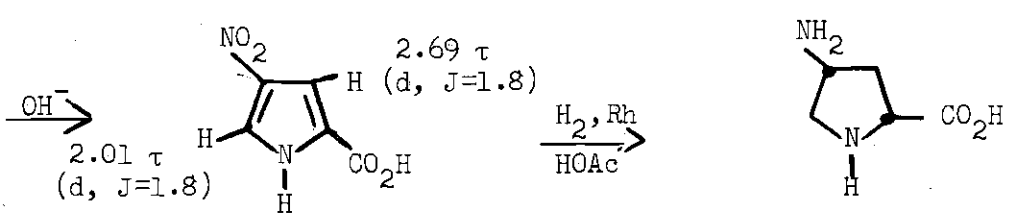
XVII

A condensation of XVII with sodium diethylaminomalonate in boiling benzene was attempted in an effort to prepare a compound which, on acid hydrolysis, would lead to trans-4-aminopyrrolidine-2-carboxylic acid (XIV). The condensation was unsuccessful and no identifiable product was obtained.

cis-4-Aminopyrrolidine-2-carboxylic acid (XXII) was successfully synthesized, using the reaction sequence shown below. Catalytic



XVIII XIX XX



XXI XXII

hydrogenation of XXI would be expected to yield preponderantly the cis isomer, XXII.

The numbers on the structures above (and in the remainder of this chapter) refer to data obtained from nuclear magnetic resonance spectra of these compounds as evidence for their correct structures. The small letters refer to the multiplicity of the peaks (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and pm = poorly resolved multiplet); the coupling constant, J, is given in cycles per second.

Ethyl 4-nitropyrrole-2-carboxylate (XX) was prepared by reacting sodium nitromalonic aldehyde (XVIII) with glycine ethyl ester (XIX) in water (23). The ester (XX) was hydrolyzed in 20 per cent potassium hydroxide solution and the tan 4-nitropyrrole-2-carboxylic acid (XXI) was recrystallized from glacial acetic acid. The acid (XXI) decomposes slightly on heating and care was taken not to overheat the compound on recrystallization. The pure colorless solid had m.p. 217°. The nitro acid had previously been hydrogenated, using a rhodium catalyst on alumina at room temperature and atmospheric pressure (9). The hydrogenation gave cis-4-aminopyrrolidine-2-carboxylic acid in about seven per cent yield. The low yield of the pure amino acid made it desirable for the synthesis to be reinvestigated in an attempt to obtain cis-4-aminopyrrolidine-2-carboxylic acid (XXII) in better yield.

Since pyrrole nuclei are not easily reduced (34) and since impurities poison the catalyst, reducing the hydrogenation yield, a highly purified sample of the starting material was necessary. Freshly recrystallized 4-nitropyrrole-2-carboxylic acid was hydrogenated in redistilled glacial acetic acid at 50 psig and room temperature, using

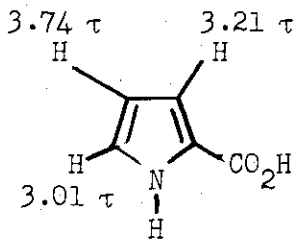
rhodium on alumina catalyst. The black solution obtained was evaporated to dryness, neutralized with IR-45(OH^-) resin, and then converted to the hydrochloride salt by ion exchange treatment, using a column of IR-45(Cl^-). The aqueous solution was decolorized, using charcoal and evaporated to dryness. This gave a partially crystalline brown solid.

The residue was recrystallized directly from water-ethanol and gave cis-4-aminopyrrolidine-2-carboxylic acid hydrochloride in 35 per cent yield. The amino acid had pK_a values of 6.60 and 9.95 in water (24) [the dissociation constant for the carboxylic acid group was not determined; proline has pK_a values 1.99 and 10.60 in water (25)]. The pK_a value 6.60 is due to the primary amino group and pK_a value 9.95 is due to the secondary amino function. The above pK_a values, the analytical data, the infrared spectrum (Fig. 1), and the nuclear magnetic resonance spectrum (Fig. 2) were consistent with cis-4-aminopyrrolidine-2-carboxylic acid as the structure of the product. The amino acid obtained is probably the cis isomer because catalytic reduction of the pyrrole nucleus would be expected to proceed in such a way that the two groups would end up on the same side of the ring.

The amino acid was further characterized by three highly crystalline derivatives: the N,N'-dibenzoyl-, m.p. 177-178°, the N,N'-bis-2,4-dinitrophenyl-, m.p. 245-248°, and the bis-2,4-dinitro-1-naphthol-7-sulfonate salt. m.p. 250-255°.

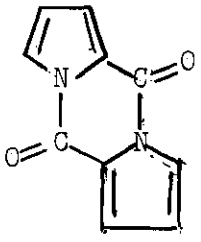
cis-3-Aminopyrrolidine-2-carboxylic acid (XXVI) was synthesized by the sequence of reactions shown below. Pyrrole-2-carboxylic acid (XXIII) was prepared and purified using the method of Maxim and his coworkers (26). Using the method of Ciamician and Silber (27), pure

pyrrole-2-carboxylic acid (XXIII) was treated with acetic anhydride to form pyrocoll (XXIV).



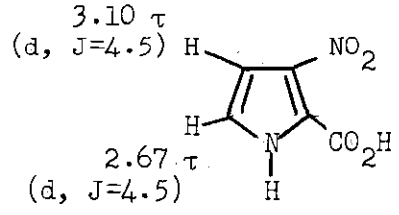
XXIII

Ac_2O



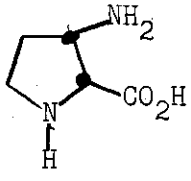
XXIV

1. HNO_3
2. NaOH



XXV

H_2, Rh
HOAc



XXVI

It had been reported in the literature that pure 3-nitropyrrole-2-carboxylic acid (XXV) (lit. m.p. 146°) could be prepared by the nitration of pyrocoll (XXIV) with nitric acid (sp. gr. 1.50) by a brief warming of the reaction mixture (23). This reaction was investigated. Instead of obtaining pure 3-nitropyrrole-2-carboxylic acid, a mixture of 3- and 4-nitropyrrole-2-carboxylic acids was obtained. The composition of the mixture was estimated from analysis of the nuclear magnetic resonance spectrum (see below) of the mixture to be about 1:1. The reaction was then investigated under different conditions; the length of the reaction time, the temperature, and the density of the nitric acid reagent used were varied. The ratio of the two isomers obtained in each run was determined from analysis of the nuclear magnetic resonance spectrum of

the mixture obtained.

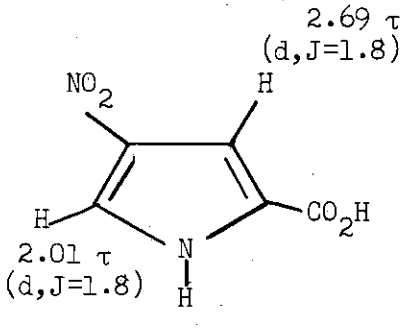
The reaction at room temperature with a 1:1 mixture of concentrated nitric acid (sp. gr. 1.42) and fuming nitric (sp. gr. 1.59) overnight was found to give the best overall yield (42 per cent) and largest amount of the 3-nitro isomer (66 per cent of the mixture). The crude product melted at 145-150° [Hale and Hoyt (23) report a melting range of 140-146° for their crude nitration product].

Hale and Hoyt (23) determined the structure of the nitropyrrole-2-carboxylic acid they obtained by thermal decarboxylation, which gave β-nitropyrrole. The isolation of β-nitropyrrole, and no α-nitropyrrole, showed that 5-nitropyrrole-2-carboxylic acid was probably not present in significant amounts in the nitration product that Hale and Hoyt obtained. this result, however, does not identify the product they obtained as 3- or 4-nitropyrrole-2-carboxylic acid or a mixture of the two. The present work indicates that a mixture of products results, whose composition depends partly on the reaction conditions.

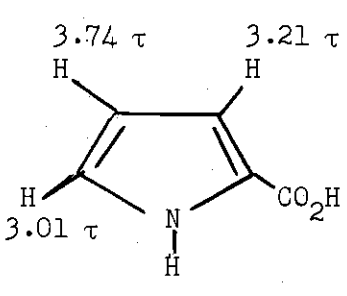
The nuclear magnetic resonance spectrum of the crude nitration product in deuterium oxide solution revealed absorptions at 2.01 τ (doublet, J = 1.8 cps, 4.8 squares), at 2.67 τ and 2.69 τ (doublet, J = 4.5 cps, and doublet, J = 1.8 cps, 13.0 squares total) and at 3.10 τ (doublet, J = 4.5 cps, 9.1 squares). In order to determine the ratio of the two isomers, the absorption positions of the protons must be assigned to both compounds. It was previously indicated that the absorption positions of pure 4-nitropyrrole-2-carboxylic acid (XXI) were 2.01 τ for the α-hydrogen and 2.69 τ for the β-hydrogen, J = 1.8 cps. This leaves the absorption values 2.67 τ and 3.10 τ for the 3-nitro isomer and clearly

indicates the 2.67 τ absorption position for the α-hydrogen and the 3.10 τ absorption position for the β-hydrogen. The ratio of the areas of the α-hydrogen in the 3-isomer to that of the β-hydrogen in the 4-isomer was 9.1 to 4.8. These data indicate that the crude nitration product was composed of about 66 per cent of 3-nitropyrrole-2-carboxylic acid (XXV) and about 34 per cent of 4-nitropyrrole-2-carboxylic acid (XXI).

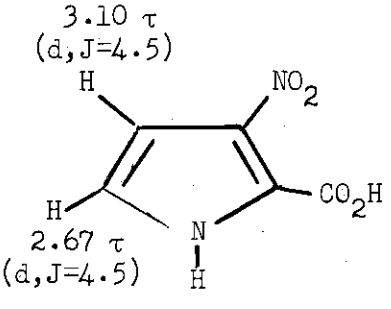
The absorption value assigned to the C_5-H in pyrrole-2-carboxylic acid (XXIII) (35) is 3.01 τ and that for the C_5-H in 4-nitropyrrole-2-carboxylic acid (XXI) is 2.01 τ. The value for C_4-H in (XXIII) is 3.74 τ and that for C_4-H in 3-nitropyrrole-2-carboxylic acid (XXV) is 3.10 τ. These data indicate that the diamagnetic anisotropic effect of a β-nitro group on an α-hydrogen of a pyrrole is about 1.00 ppm, and that of a β-nitro group on a β'-hydrogen of a pyrrole is about 0.64 ppm. The difference between the C_3-H in (XXIII) and the C_4-H in (XXIII) is 0.53 ppm, which suggests that the diamagnetic anisotropic effect of an α-carboxyl group on a β-hydrogen is about 0.52 ppm.



XXI



XXIII



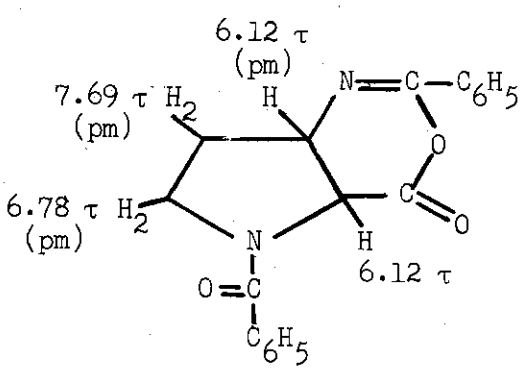
XXV

The coupling constants for pyrrole-2-carboxylic acid (XXIII) are: $J_{3,5}$ = 1.6, $J_{4,5}$ = 2.6, and $J_{3,4}$ = 3.7 cps. In general, pyrroles show

$J_{2,3}$ = 2.0-2.6, $J_{2,4}$ = 1.5-2.2, $J_{2,5}$ = 1.8-2.3, and $J_{3,4}$ = 2.8-4.0 cps (35). The value $J_{3,5}$ = 1.8 cps for 4-nitropyrrole-2-carboxylic acid (XXI) is consistent with the above data but the value $J_{4,5}$ = 4.5 cps for the 3-nitro compound (XXV) is unusually large. This value is closer to that for β,β' coupling ($J_{3,4}$), as might be expected for 5-nitropyrrole-2-carboxylic acid. That the compound obtained is 3-, and not 5-nitropyrrole-2-carboxylic acid is shown by the fact that Hale and Hoyt (23) decarboxylated the substance, which gave, at least predominantly, β-nitropyrrole. In addition, as described below, the substance was reduced and gave cis-3-aminopyrrolidine-2-carboxylic acid (5-aminopyrrolidine-2-carboxylic acid, being the nitrogen analog of an aldehyde hydrate, would not be expected to be stable), which was characterized by an anhydro dibenzoyl derivative, characteristic of β-amino acids.

The crude nitration mixture was recrystallized three times from benzene (m.p. 146-148°) and the nuclear magnetic resonance spectrum of the product was analyzed. The spectrum revealed the presence of 95 per cent 3-nitropyrrole-2-carboxylic acid and five per cent 4-nitropyrrole-2-carboxylic acid. The purified 3-nitropyrrole-2-carboxylic acid (XXV) was hydrogenated in redistilled glacial acetic acid at room temperature and 50 psig, using a rhodium on alumina catalyst. The hydrogenated product could not be crystallized because of its hygroscopic nature. However, the infrared spectrum (Fig. 3) and the nuclear magnetic resonance spectrum (Fig. 4) of the crude product were consistent with the structure (XXVI) for cis-3-aminopyrrole-2-carboxylic acid. The amino acid was characterized by a highly crystalline derivative, the bis-2,4-dinitronaphthol-7-sulfonate salt, m.p. 229-231°.

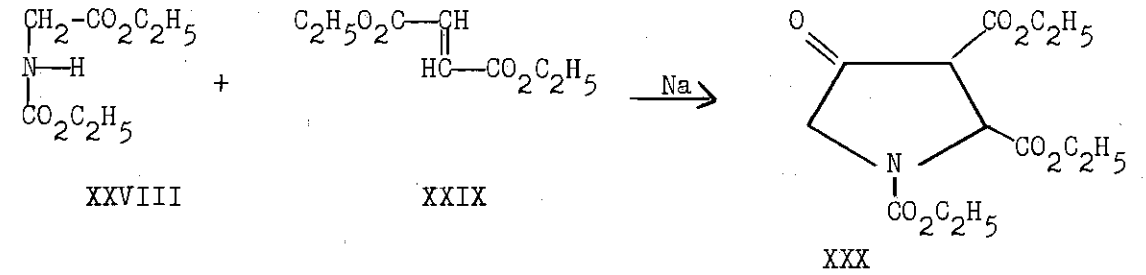
When the amino acid was treated with benzoyl chloride in sodium hydroxide solution, in an effort to obtain the N,N'-dibenzoyl derivative, a precipitate was instantly formed, unexpectedly, in the basic medium. Elemental analysis of the crystalline derivative, obtained in 67 per cent yield, indicated a molecular formula of $C_{19}H_{16}N_2O_3$, one molecule of water less than the formula anticipated for the N,N'-dibenzoyl derivative. It is reported in the literature that benzoylation of many β-amino acids give benzoyl derivatives that are easily dehydrated to stable oxazines (35,37). Accordingly, and in agreement with the analytical data obtained, the dibenzoyl derivative was assigned structure (XXVII). This result further indicated that the carboxylic acid group and the amino group in the amino acid (XXVI) are cis, as was anticipated from the catalytic reduction. The assignment of the peaks observed in the nuclear magnetic resonance spectrum of a trifluoroacetic acid solution of XXVII are shown below.



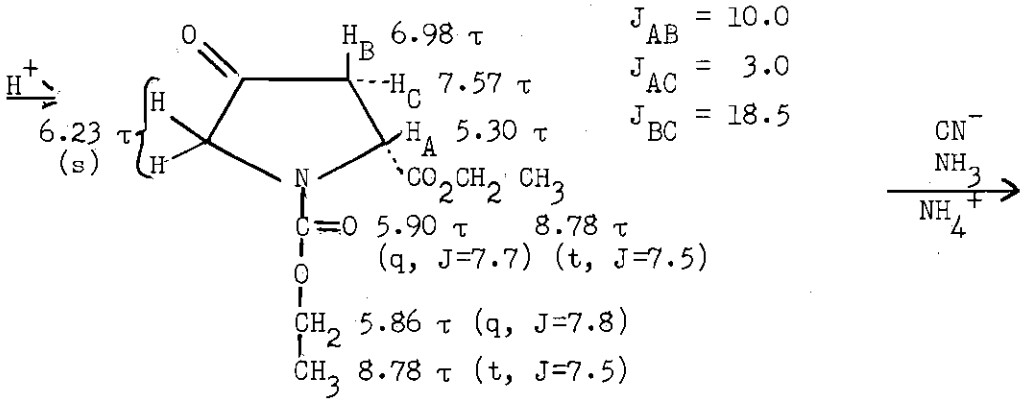
XXVII

It has been reported that proline can be oxidized by periodate ion in the presence of mercuric chloride to give the mercuric chloride complex of Δ^1-pyrroline (38). A plan to synthesize one of the proposed

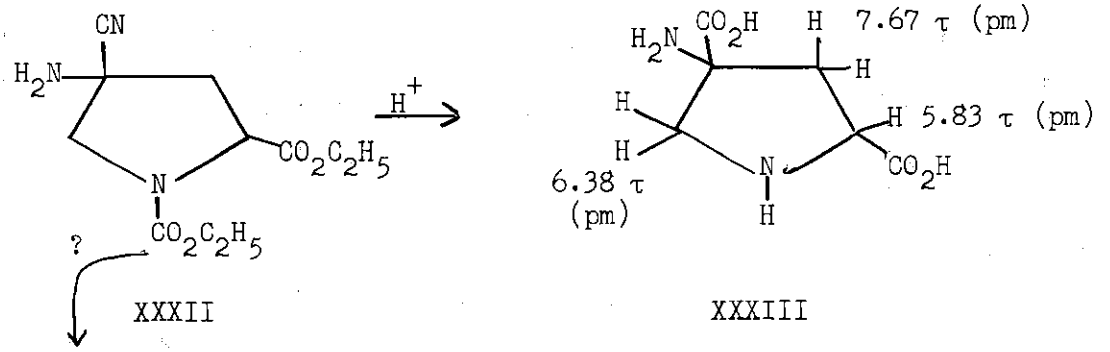
structures for viomycidine (VIII) was proposed as shown below.



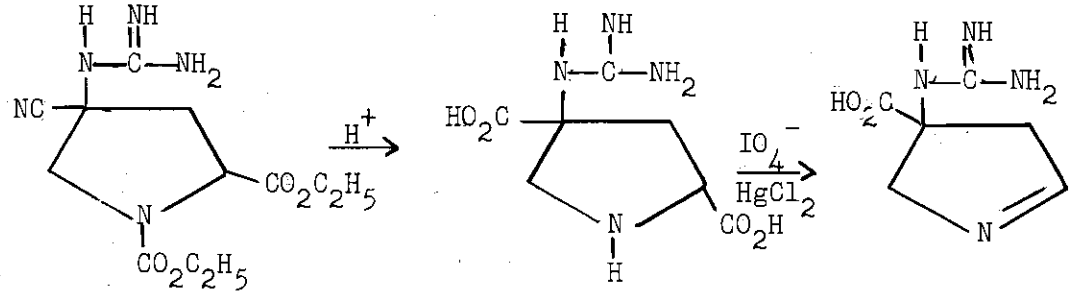
XXVIII XXIX XXX



XXXI



XXXII XXXIII



1,2-Dicarbethoxy-4-pyrrolidone (XXXI) was prepared from N-carbethoxyglycine ethyl ester (XXVIII) and diethyl fumarate (XXIX) through 1,2,3-tricarbethoxy-4-pyrrolidone (XXX), using the method of Kuhn and Osswald (28). The assigned nuclear magnetic resonance peaks are shown above.

The Strecker reaction with 1,2-dicarbethoxy-4-pyrrolidone (XXXI) was examined to determine the best reaction time. It was found that the largest weight of basic fraction was obtained after six days and the strongest nitrile band (4.45 μ) in the infrared spectrum was present after this time. The basic fraction contained 4-cyano-4-amino-1,2-dicarbethoxypyrrolidine (XXXII). The nuclear magnetic resonance spectrum of the crude basic fraction was similar to that of starting ketone (XXXI) except that the methylene protons at C_5 were, as expected, nonequivalent and formed an AB pattern centered at 6.23 τ.

Acid hydrolysis of 4-cyano-4-amino-1,2-dicarbethoxypyrrolidine (XXXII) resulted in the formation of 4-aminopyrrolidine-2,4-dicarboxylic acid (XXXIII). Elemental analysis, the infrared spectrum (Fig. 5), and the nuclear magnetic resonance spectrum (Fig. 6) were consistent with the assigned structure for the new amino acid. From the nuclear magnetic resonance spectrum it appeared probable that the substance is a mixture of stereoisomers. The amino acid was further characterized by two highly crystalline derivatives: the N,N'-bis-dinitrophenyl, m.p. 247-250°, and bis-2,4-dinitro-1-naphthol-7-sulfonate salt, m.p. 250-253°.

Recently, 1-aminocyclopentanecarboxylic acid and some of its peptide and dipeptide derivatives were reported to have antitumor activity (39). Since 4-aminopyrrolidine-2,4-dicarboxylic acid (XXXIII) also has a 1-aminocyclopentanecarboxylic acid moiety, it was thought

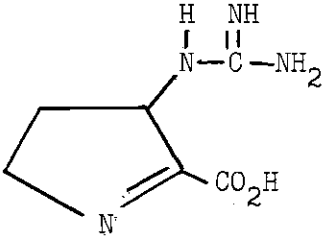
that the compound might have antitumor activity. The amino acid (XXXIII) also has a proline group, which is fairly common in some enzymes, and could result in enhanced biological activity. However, biological testing showed that the amino acid did not exhibit antiviral or antibacterial activity (40). At the same time cis-4-aminopyrrolidine-2-carboxylic acid (XXII) was also tested and showed no biological activity.

In an attempt to prepare the guanidine derivative of the aminonitrile (XXXII) the compound was treated with an aqueous solution of 1-guanyl-3,5-dimethylpyrazole in the presence of triethylamine (41). The Weber-positive product was hydrolyzed with acid, chromatographed on a carbon column, and recrystallized from ethyl acetate. The Weber-positive compound obtained gave a negative Sakaguchi test, which indicated that the compound was not a monosubstituted guanidine. Elemental analysis revealed a molecular formula of $C_3H_9N_3O_2$, which is the formula of guanidine acetate. The R_F values of the compound in three solvent systems, the infrared spectrum, and the nuclear magnetic resonance spectra in deuterium oxide and trifluoroacetic acid were identical with those of guanidine acetate. At that stage of the research, structure VIII for viomycidine was proved to be incorrect and the synthetic work was abandoned. The source of guanidine acetate is not clear in the reaction sequence but it is thought that it might have formed during recrystallization of the produce from ethyl acetate.

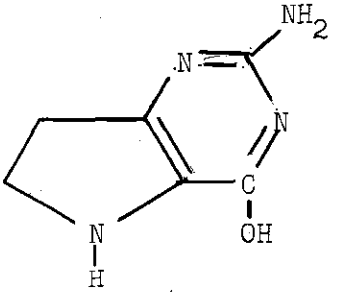
The Structure of Viomycidine

Structure V had been proposed for viomycidine on the basis of the available chemical and physical data (16). This structure was also

accepted by Johnson (15) and on the basis of other data obtained from a dipeptide he isolated from the partial acid hydrolysate of viomycin, Johnson postulated the cyclic structure (VII) as the structure of viomycidine as it occurs in the viomycin molecule.



V

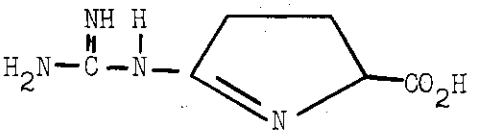


VII

Viomycidine is obtained from viomycin by acid hydrolysis. If structure (VII), proposed by Johnson, is considered to be the correct structure of viomycidine in the viomycin molecule, acid hydrolysis would certainly give racemic viomycidine and not an optically active substance. Since crystalline viomycidine hydrochloride is optically active, the cyclic structure (VII) proposed by Johnson is incorrect and hence structure V for viomycidine is also incorrect. The ultraviolet spectrum of viomycin clearly shows that the guanidine group is involved in the chromophore, and so it seems very unlikely that the carbon atom to which the guanidine group of viomycidine is attached can be asymmetric. The guanidine group of viomycidine shows a differential ultraviolet spectrum (λ_{max}, 222 mμ, ε 1690 using pH 13.1 versus pH 9.0), which suggests that the guanidine group is attached to an unsaturated carbon atom (24). Thus the carbon atom to which the carboxylic acid group is attached, and not that to which

the guanidine group is attached, is the asymmetric carbon atom in the viomycidine molecule.

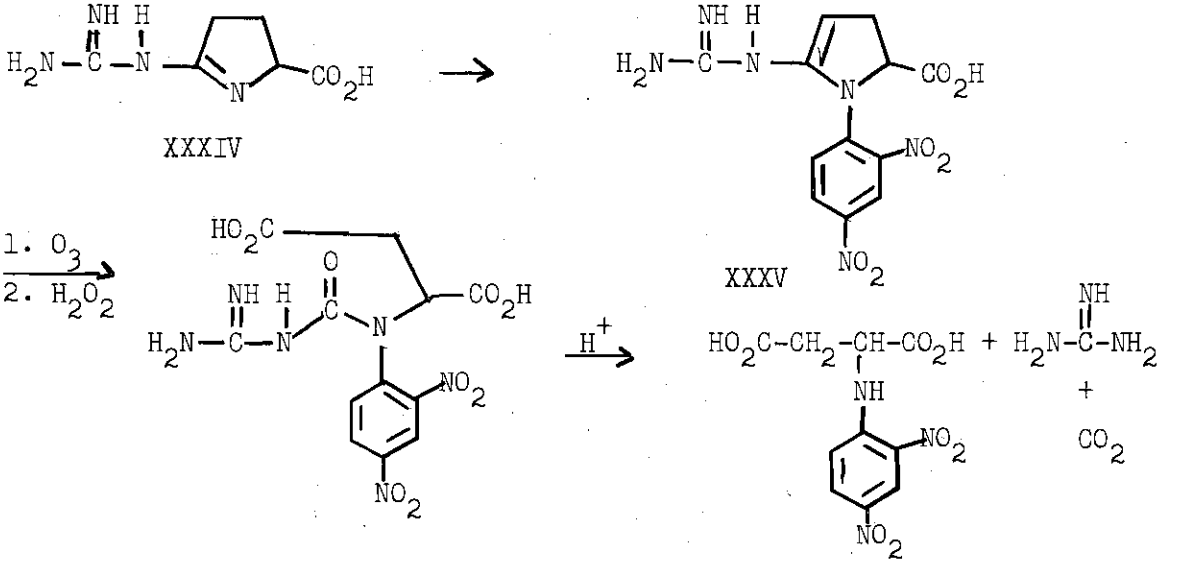
Based on the above and other data, the structure 2-guanido-Δ^1-pyrroline-5-carboxylic acid (XXXIV) was suggested for viomycidine. This



XXXIV

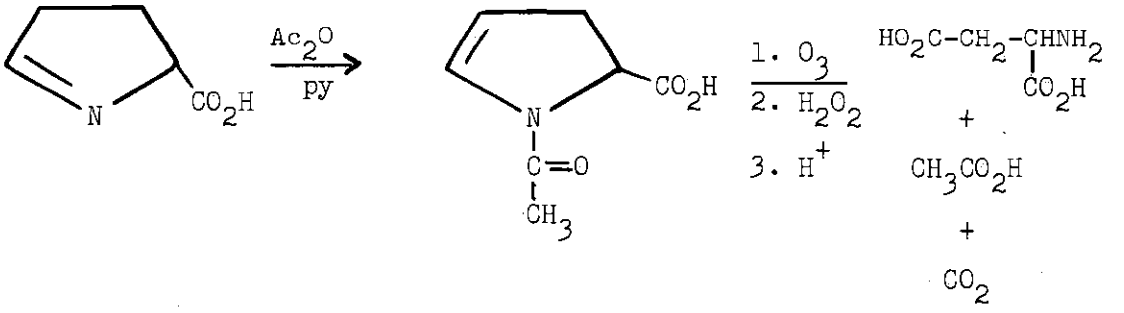
structure accounts for the presence of the guanidine group on an unsaturated carbon atom, the presence of a Δ^1-pyrroline moiety, and the presence of an asymmetric carbon to which the carboxylic acid group is attached.

It had been reported in the literature that alkylation or acetylation of Δ^1-pyrrolines give, depending on the reaction condition, either N-substituted Δ^2-pyrrolines or N-substituted aminoketones, or a mixture of both (42,43,44). Accordingly, 2,4-dinitrophenylviomycidine was prepared and assigned the Δ^2-pyrroline structure (XXXV).



XXXV

In order to prove the proposed structure for viomycidine (XXXIV), the ozonolysis of 2,4-dinitrophenylviomycidine was investigated in an effort to isolate 2,4-dinitrophenylaspartic acid, as it is shown in the above reaction sequence. This was based on a recent report by Zbiral (45), in which Δ^1-pyrroline-5-carboxylic acid was acetylated and ozonized. The ozonide product was oxidized with hydrogen peroxide and the product was hydrolyzed in acid. Aspartic acid was obtained as shown in the accompanying reaction sequence.



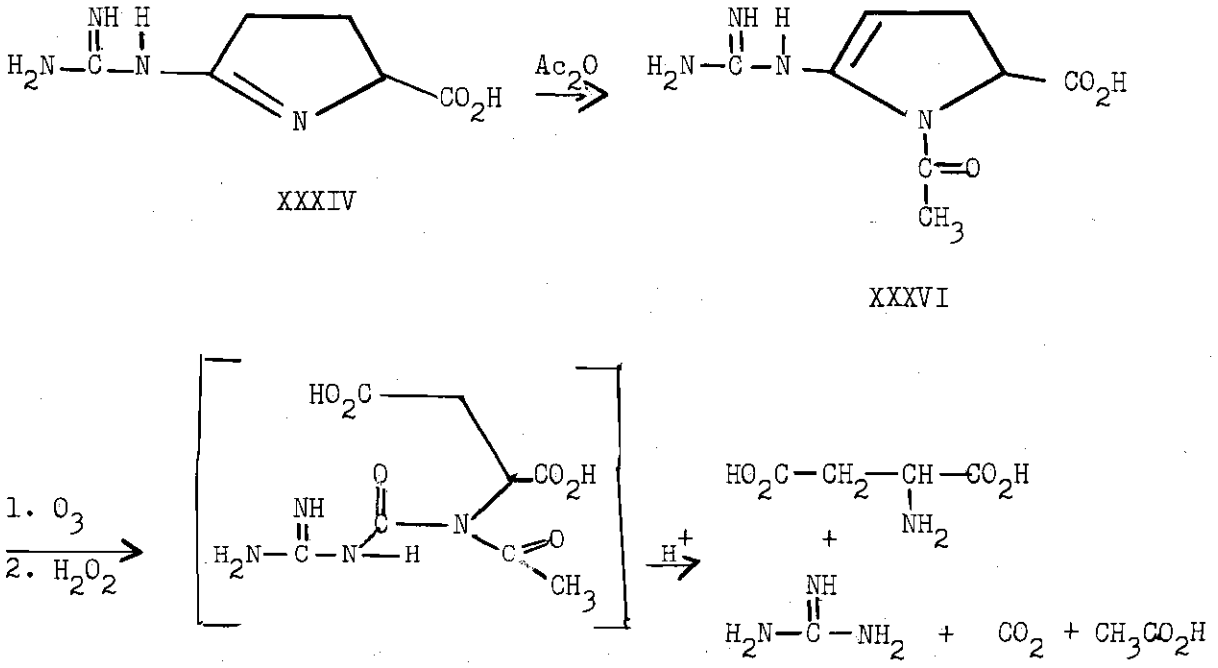
2,4-Dinitrophenylviomycidine (XXXV) was prepared by reacting viomycidine (XXXIV) with 2,4-dinitrofluorobenzene in an ethanol-water mixture in the presence of an excess of sodium bicarbonate (25). When 2,4-dinitrophenylviomycidine was ozonized in cold 80 per cent formic acid and the ozonized product was oxidized with hydrogen peroxide, the product formed showed two yellow spots by TLC analysis. One of the spots corresponded to 2,4-dinitroaniline. The ozonized product was then hydrolyzed with concentrated (ca 12 N) hydrochloric acid in a pressure bottle at 95°. Analysis of the hydrolysate by paper chromatography and by thin layer chromatography revealed guanidine, 2,4-dinitroaniline and an unknown Weber-positive compound that was not identified.

No 2,4-dinitrophenylaspartic acid or aspartic acid was detected. It was suspected that the 2,4-dinitrophenyl derivative was attacked by the ozone, destroying any 2,4-dinitrophenylaspartic acid formed. In order to prove this assumption, 2,4-dinitrophenylaspartic acid was ozonized under the same conditions used for the ozonolysis of 2,4-dinitrophenylviomycidine. TLC analysis of the product indicated the presence of 2,4-dinitroaniline as the main component; no 2,4-dinitrophenylaspartic acid remained.

Since 2,4-dinitrophenylaspartic acid did not survive the ozonolysis condition, it was thought possible that the double bond in 2,4-dinitrophenylviomycidine could be oxidized with potassium permanganate if 2,4-dinitrophenylaspartic acid was stable to the oxidation conditions. However, when 2,4-dinitrophenylaspartic acid was treated with acidic potassium permanganate solution, the permanganate solution was decolorized within a few minutes. A yellow solid separated when the solution was cooled; it showed the same R_F value as 2,4-dinitroaniline by TLC analysis. The solid was isolated and was found to have the same melting point as an authentic sample of 2,4-dinitroaniline; no depression in melting point was observed when a mixture melting point with the latter was determined. It was concluded from these experiments that neither ozone nor permanganate could be used to oxidize 2,4-dinitrophenylviomycidine to 2,4-dinitrophenylaspartic acid because the latter, if produced, was oxidized by these reagents.

In a further effort to ozonize the double bond in a derivative of viomycidine and hence obtain evidence for its structure, a sequence of reactions similar to that used by Zbiral (45) was proposed.

Acetylviomycidine (XXXVI) was prepared from viomycidine, using

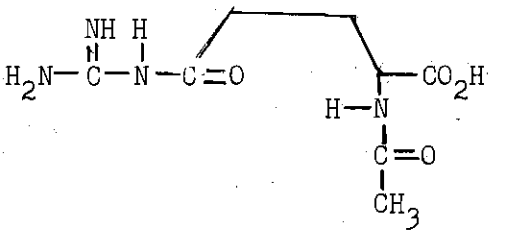


XXXIV

XXXVI

cold aqueous acetic anhydride by the method of Miller (11). The crude product was ozonized, oxidized with hydrogen peroxide, and hydrolyzed by acid, according to the method of Zbiral (45). The hydrolysate contained, as indicated by paper chromatography, a mixture of guanidine, viomycidine, aspartic acid, and an unidentified ninhydrin-positive, Weber-negative compound. The colors of the spots corresponding to both aspartic acid and guanidine were very weak, an indication that the acetyl derivative probably has a different structure than XXVI or it might be a mixture of XXXVI and XXXVII, as was previously suggested (11).

Viomycidine was acetylated with acetic anhydride and pyridine at steam-bath temperature. The crude acetyl derivative was ozonized and worked up as mentioned above. A black residue remained. The residue was analyzed by paper chromatography and was found to be composed of



XXXVII

aspartic acid, viomycidine, and guanidine. The color intensity of the spot corresponding to viomycidine indicated that the latter was a minor component in the mixture. Both the aspartic acid and the guanidine components had the same R_F values as those of authentic samples in three solvent systems. The crude residue was reacted with 2,4-dinitrofluorobenzene in an effort to obtain crystalline 2,4-dinitrophenylaspartic acid. The product was a brown-yellowish oil that could not be recrystallized. The oil was analyzed by TLC. At least four different compounds, one of which had the same R_F value as an authentic sample of 2,4-dinitrophenylaspartic acid, were present. The whole mixture was chromatographed on thick TLC plates and the band corresponding to 2,4-dinitrophenylaspartic acid was removed and eluted with five per cent formic acid in acetone. The yellow compound obtained had the same R_F value as 2,4-dinitrophenylaspartic acid in nine solvent systems. However, all attempts to obtain the derivative in crystalline form failed.

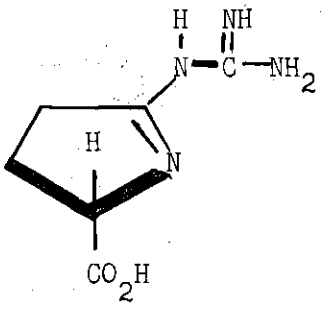
A portion of the hydrolysate of the ozonized product was chromatographed on thick paper in BAW solvent. The band corresponding to aspartic acid was eluted from the paper and then chromatographed on a carbon column. The white solid obtained was crystallized from water and afforded

a crystalline compound in 53 per cent yield. The crystalline product showed no rotation in 1 N hydrochloric acid; its infrared spectrum (potassium bromide pellet) was superimposable on that of an authentic sample of DL-aspartic acid but different from that of L-aspartic acid, and its nuclear magnetic resonance spectrum in 0.5 N hydrochloric acid was identical to that of DL-aspartic acid in the same solvent. These data show that the proposed structure, 2-guanido-Δ^1-pyrroline-5-carboxylic acid (XXXIV) is the structure of viomycidine.

Since viomycidine is optically active, it was thought that the aspartic acid obtained would be optically active. If this had been the case, the absolute configuration of viomycidine would have been determined with assurance. Racemization is attributed to the presence of hot pyridine at one stage of the reaction sequence. The configuration of viomycidine could be determined if acetylviomycidine could be prepared under non-racemizing conditions.

According to the Clough-Lutz-Jirgensons rule, acidification of an aqueous solution of an L-α-amino acid causes the molecular rotation to assume a more positive value; on the other hand, acidification of an aqueous solution of a D-α-amino acid causes the rotation to become more negative (25). Since viomycidine was found to have an $[M]_D = -37.6^0$ in water and -10.3^0 in 2 N hydrochloric acid (9), $\Delta[M]_D = +27.3^0$, the compound has the configuration shown in XXXVIII.

X-ray crystallographic (46) examination of viomycidine hydrochloride revealed that it was orthorhombic and belonged to space group $C222_1$ (47). The unit cell was found to contain eight molecules and to have the dimensions 9.31 Å x 12.15 Å x 14.81 Å. The density of



XXXVIII

crystalline viomycidine hydrochloride was observed to be 1.60 (calculated, 1.64).

Studies on the Cyemistry of Viomycin

In an attempt to obtain a partial hydrolysate of viomycin and isolate intermediate peptides, the study of which might give further information on the arrangement of the different amino acid fragments in the viomycin molecule, the antibiotic was treated with the enzyme pronase at pH 8.0 and room temperature for 15 days. Papergram analysis of the reaction solution, however, indicated that viomycin was not attacked by the enzyme and no hydrolysis had taken place.

Because analytical data for viomycin, desureaviomycin, and viomycinic acid are in better agreement with formulas containing one nitrogen atom less than the generally accepted formula, it was thought possible that the ammonia observed in viomycin hydrolysate could be an artifact and arise from acid catalyzed degradation of the observed hydrolysis products of viomycin.

Viomycin was hydrolyzed with 6 N hydrochloric acid at 95° for

six hours and the whole hydrolysate was analyzed by an amino acid analyzer (48). The results revealed an actual ratio of 1.00 mole of urea, 0.110 mole of ammonia, and 0.145 mole of viomycidine. When urea was hydrolyzed under the same conditions, 0.073 mole of ammonia was released per mole of urea. The release of a small amount of ammonia from each individual amino acid produced by the hydrolysis is possible. Thus, ammonia is not a real hydrolysis product of viomycin; its release in the hydrolysis is an artifact.

Mason (5) found that when viomycin was hydrolyzed with hot 0.43 N barium hydroxide, the hydrolysate showed a papergram pattern with a spot corresponding to alanine. Since alanine does not occur in the acid hydrolysate of viomycin, the basic hydrolysis was repeated in an attempt to isolate and identify this unknown fragment.

Viomycin hydrochloride was hydrolyzed with 0.43 N barium hydroxide at 95° for three days. The hydrolysis mixture was chromatographed on thick paper in PW solvent. The sections corresponding to the presumed alanine fraction were cut out and eluted with water. The compound obtained showed by papergram analysis only one spot, which corresponded to alanine, but the residue was slightly colored. The unknown was then chromatographed on a carbon column. The colorless solid, obtained in 22 per cent yield (based on the formula $C_{23}H_{38}H_{12}O_9 \cdot 3HCl$ for viomycin hydrochloride), showed no rotation in 5.0 N hydrochloric acid solution. The nuclear magnetic resonance spectrum of the compound in deuterium oxide was identical with a spectrum of an authentic sample of alanine determined in a similar solution. A sample of the natural DL-alanine was treated with 2,4-dinitrofluorobenzene in order to obtain

the 2,4-dinitrophenyl derivative. The reaction afforded a few milligrams of yellow crystals that melted at 155-160°. The 2,4-dinitrophenyl derivative of an authentic sample of DL-alanine melted at 173-174°. The wide range of melting point displayed by the derivative from the natural DL-alanine indicated that the product was not pure enough for a mixed melting point determination. However, when the infrared spectrum of this derivative was determined, as a potassium bromide pellet, it was found to be identical with a spectrum of the 2,4-dinitrophenyl derivative of an authentic sample of DL-alanine. The presence of DL-alanine in the basic hydrolysate of viomycin and its absence from the acid hydrolysate is attributed to the attack of the base on the serine fragment, released from viomycin during the hydrolysis, to give the resulting alanine. Wieland and Wirth (49) studied the hydrolysis of serine with hot saturated barium hydroxide at 110° for 15 hours in a sealed tube. They reported that the product showed, by paper chromatography, the presence of serine, glycine, and alanine. The hydrolysis of serine was repeated under the same condition used for the hydrolysis of viomycin above. Papergram analysis of the hydrolysate indicated the presence of serine and alanine. DL-Alanine was presumably obtained from the L-serine, in the viomycin hydrolysate, by base-catalyzed racemization of the latter.

When viomycidine was degraded with barium hydroxide at 95° for 77 hours, pyrrole-2-carboxylic acid was isolated in 22 per cent yield. (11). The barium hydroxide hydrolysate of viomycin hydrochloride at 95° after 73 hours was passed over an IR C-50(H^+) column. The eluate from this column contained only acidic and neutral compounds. From this eluate

a pyrrole acid was obtained in three per cent yield. This compound and pyrrole-2-carboxylic acid were found to have identical infrared and ultraviolet spectra, the same R_F value in n-BAW and the same color when the chromatogram was sprayed with Erlich's reagent.

In another run of the barium hydroxide hydrolysis for three days, the reaction mixture was acidified with sulfuric acid and distilled. The distillate was titrated with potassium hydroxide (0.25 mole of base per 1.24 mole of viomycin was needed) and the neutral solution was evaporated to dryness. The white solid obtained had a nuclear magnetic resonance spectrum identical to that of sodium acetate. The salt was obtained in 20 per cent yield.

The basic hydrolysate was also tested for the presence of oxalic acid by treating the acidified hydrolysate with an excess of IR-45(OH^-). The resin was then washed with hydrochloric acid to elute any acidic compound, and the eluate was evaporated to dryness. This residue gave a negative aniline blue test for oxalic acid (18).

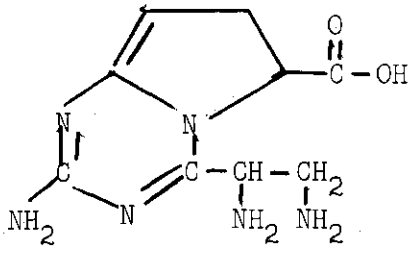
In an effort to determine the amount and the origin of ammonia released from viomycin on barium hydroxide hydrolysis, viomycin hydrochloride and some of its known hydrolysis products were hydrolyzed with 0.43 N barium hydroxide at 95°. The amount of ammonia evolved was determined versus time. The results obtained are shown in Table 8 and Fig. 7. L-Serine and α,β-diaminopropionic acid released only a negligible amount of ammonia during four days of hydrolysis. On the other hand, urea was completely converted to ammonia and carbon dioxide in about 70 hours, and viomycidine released almost three moles of ammonia within the same period of time. This clearly indicates that practically

all the ammonia liberated from viomycin comes from the urea and the guanidine function in viomycin. When the curve for the urea hydrolysis is subtracted from that for viomycin, a new curve results which levels off after about 20 hours of hydrolysis at a value of approximately one mole of ammonia per mole of viomycin. This shows that during the first 20 hrs. one mole of ammonia was relatively easily released from the viomycin molecule in excess of that arising from the urea function. This mole of ammonia is postulated to have been released from the viomycidine precursor by transforming the "guanidine unit" to a "ureido unit."

It has been already indicated that Johnson (15) had postulated structure (VII) for the viomycidine moiety in the viomycin molecule. This unit, which was proposed to be responsible for the ultraviolet chromophore of viomycin, was postulated to be the part structure of a dipeptide (VI) that Johnson isolated from partial acid hydrolysis of viomycin. This dipeptide showed a similar ultraviolet spectrum to that of viomycin and gave viomycidine and α,β-diaminopropionic acid on acid hydrolysis. The 2,4-dinitrophenyl derivative of the dipeptide released viomycidine and bis-2,4-dinitrophenyl-α,β-diaminopropionic acid when hydrolyzed with acid, which indicated the presence of two primary amino groups in the diaminopropionic acid fragment.

Structure (VI), which would contain the chromophoric group of viomycin, could not account for the low field proton (1.93 τ) present in the nuclear magnetic resonance spectrum of the antibiotic (6) and, as already indicated, it will not give optically active viomycidine on acid hydrolysis.

In light of the correct structure for viomycidine (XXXIV), a new structure (XXXIX) is postulated for the dipeptide. This structure,



XXXIX

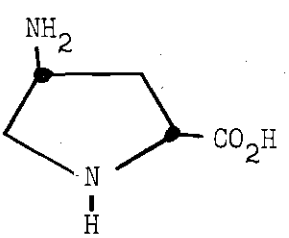
besides satisfying all of Johnson's chemical data, has a chromophoric unit that could be responsible for the ultraviolet spectrum, has an olefinic proton that could absorb in the low field region in the nuclear magnetic resonance spectrum, and upon acid hydrolysis would release optically active viomycidine.

Since carbon dioxide was not released from viomycin on mild barium hydroxide treatment, it is postulated that carbon dioxide probably is not present as a urethane group in viomycin (50).

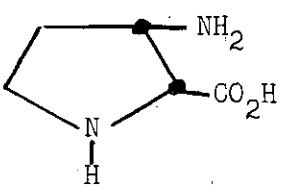
CHAPTER IV

CONCLUSIONS

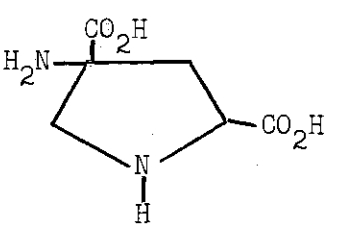
cis-4-Aminopyrrolidine-2-carboxylic acid (XXII), cis-3-aminopyrrolidine-2-carboxylic acid (XXVI), and 4-aminopyrrolidine-2,4-dicarboxylic acid (XXXIII) have been synthesized. Analytical data and infrared and nuclear magnetic resonance spectra were consistent with the structures of the amino acids. The amino acids were characterized by the preparation of several crystalline derivatives. The amino acids XXII and XXXIII were found to have no antiviral or antibacterial activity.



XXII

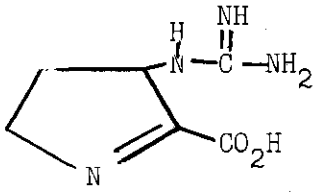


XXVI

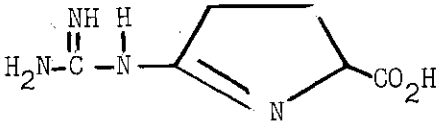


XXXIII

A previously suggested structure (V) for viomycidine has been rejected on the basis of data published by Johnson (15), which was based on structure (V) as the correct structure for viomycidine. Accordingly, and on the basis of the available chemical and physical data, a new structure (XXXIV) was postulated for viomycidine. This structure was proved to be the correct structure of viomycidine by the isolation and identification of DL-aspartic acid, as anticipated, from the ozonolysis and hydrolysis of acetylviomycidine. The recemization of the



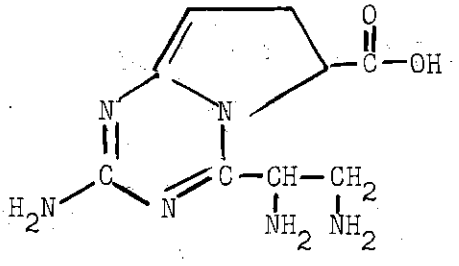
V



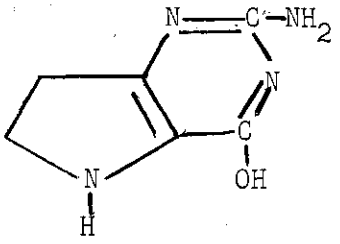
XXXIV

optically active center of viomycidine during the process is attributed to the presence of hot pyridine at one stage of the reaction sequence. It is suggested that the reaction be repeated, using nonracemizing conditions for preparing the acetyl derivative of viomycidine in order to obtain optically active aspartic acid and hence determine the configuration of viomycidine.

This correct structure for viomycidine suggests a new chromophoric group (XXXIX) in the viomycin molecule rather than the one suggested by Johnson (VII). This new chromophoric unit, besides explain-



XXXIX



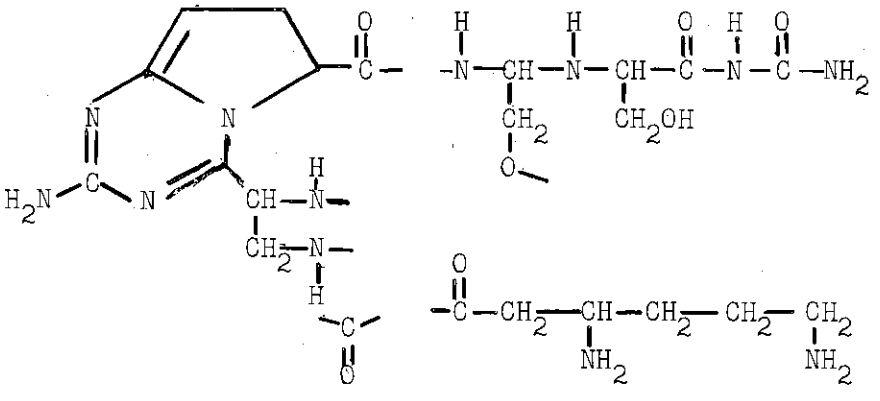
VII

ing all the data obtained from VII, accounts for the presence of a low field proton in the nuclear magnetic resonance spectrum of viomycin and for the release of optically active viomycidine. Neither fact could be

accounted for by structure (VII).

The isolation and identification of DL-alanine from the barium hydroxide hydrolysis of viomycin is attributed to the transformation of the serine fragments, released from the antibiotic, to alanine by base during the hydrolysis (49). Interpretation of the rate of release of ammonia from the barium hydroxide hydrolysis of viomycin and some of its components indicate the release of one mole of ammonia from viomycin in excess of that released from the urea fragment in the first twenty hours of hydrolysis. This easily released ammonia is attributed to the change of a "guanidine unit" to a "ureido unit" in the viomycin chromophore. It was also concluded from the quantitative amino acid analysis of the acid hydrolysate of viomycin that ammonia is an artifact in the acid hydrolysate of viomycin. Since carbon dioxide was not released from the antibiotic by mild base hydrolysis, it was postulated that carbon dioxide does not exist as a urethane moiety in viomycin.

In summary, the structural formula of viomycin is composed of the following partial structures:



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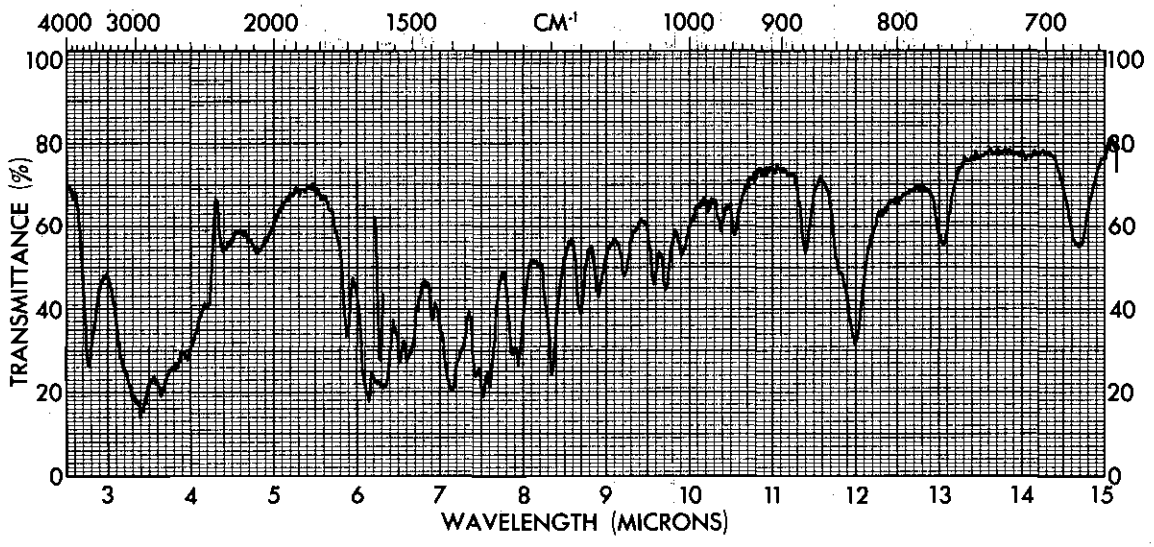

Figure 1. Infrared Spectrum of cis-4-Aminopyrrolidine-2-carboxylic Acid Hydrochloride.

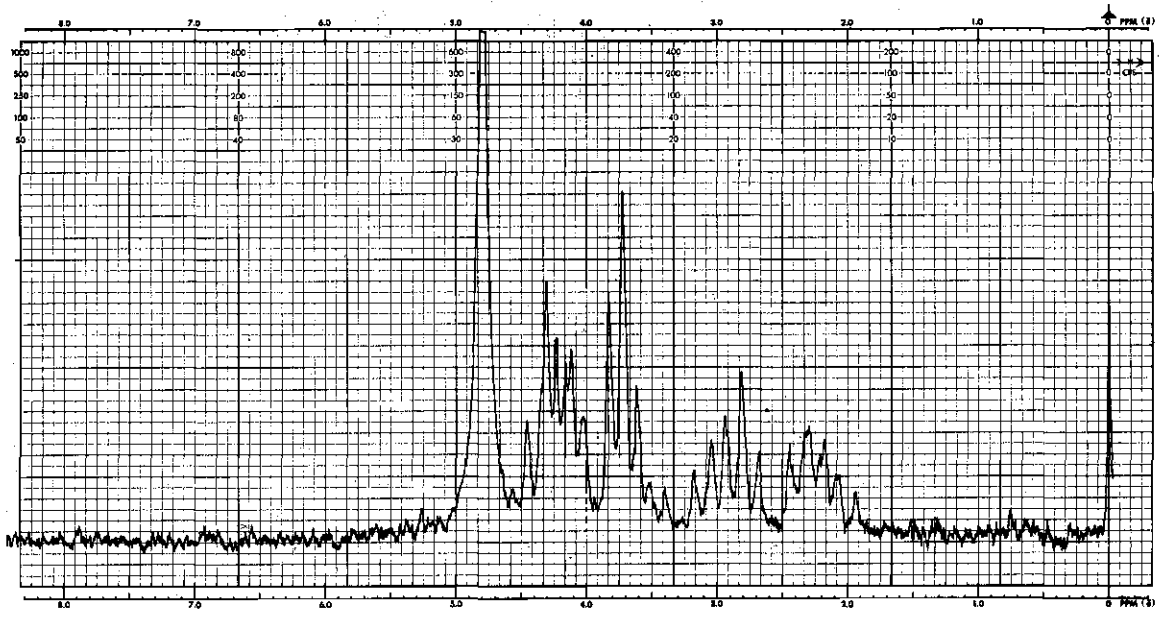

Figure 2. Nuclear Magnetic Resonance Spectrum of cis-4-Aminopyrrolidine-2-carboxylic Acid Hydrochloride in Deuterium Oxide Solution.

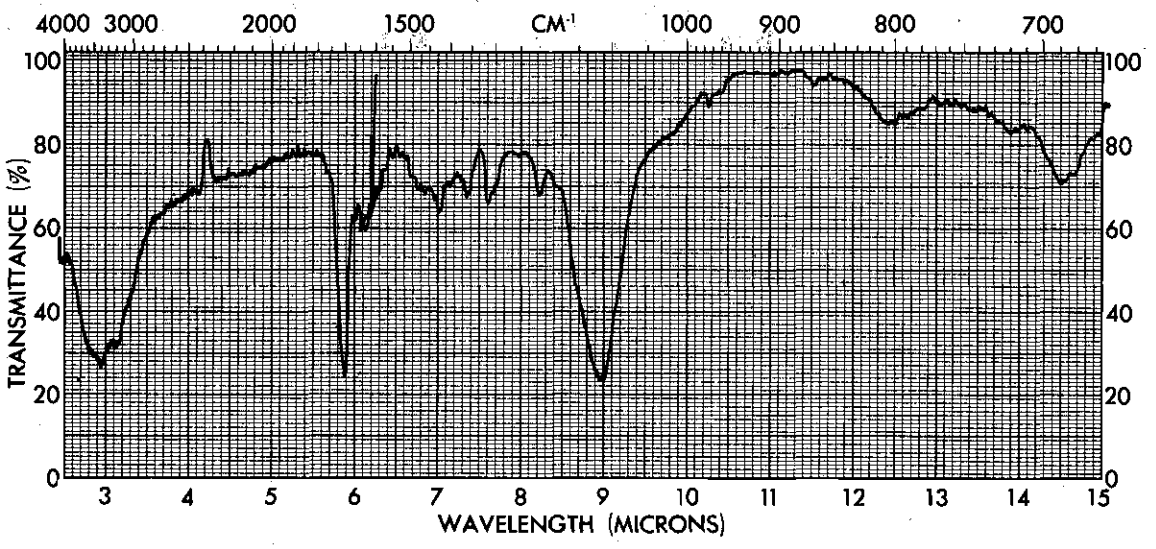

Figure 3. Infrared Spectrum of cis-3-Aminopyrrolidine-2-carboxylic Acid Sulfate.

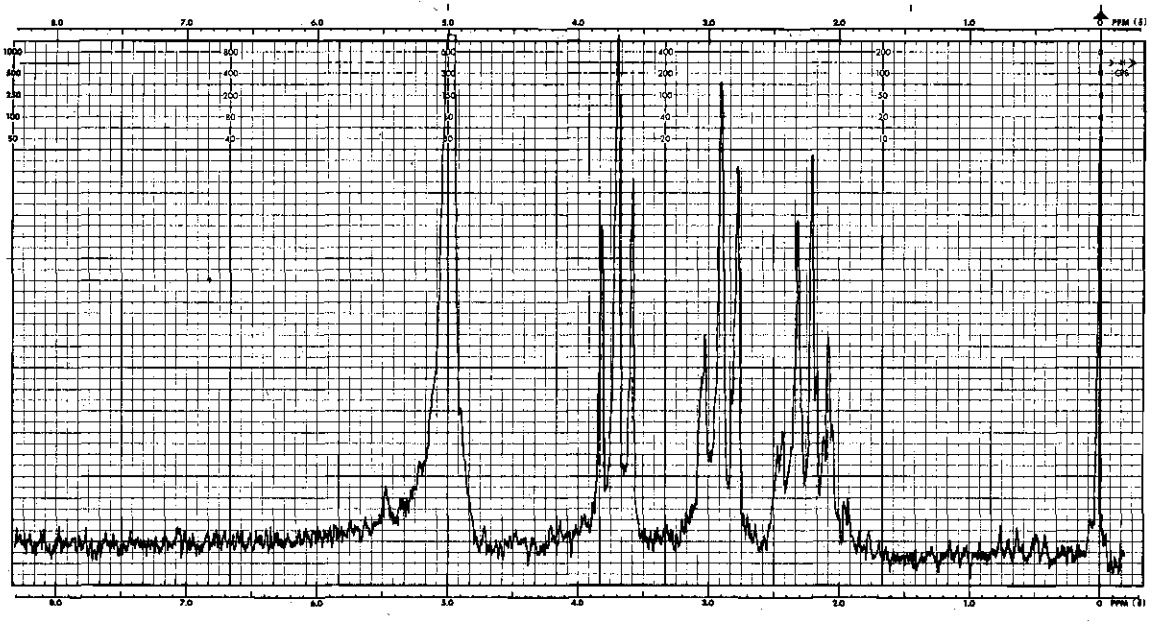

Figure 4. Nuclear Magnetic Resonance Spectrum of cis-3-Aminopyrrolidine-2-carboxylic Acid Sulfate in Deuterium Oxide and Sulfuric Acid Solution.

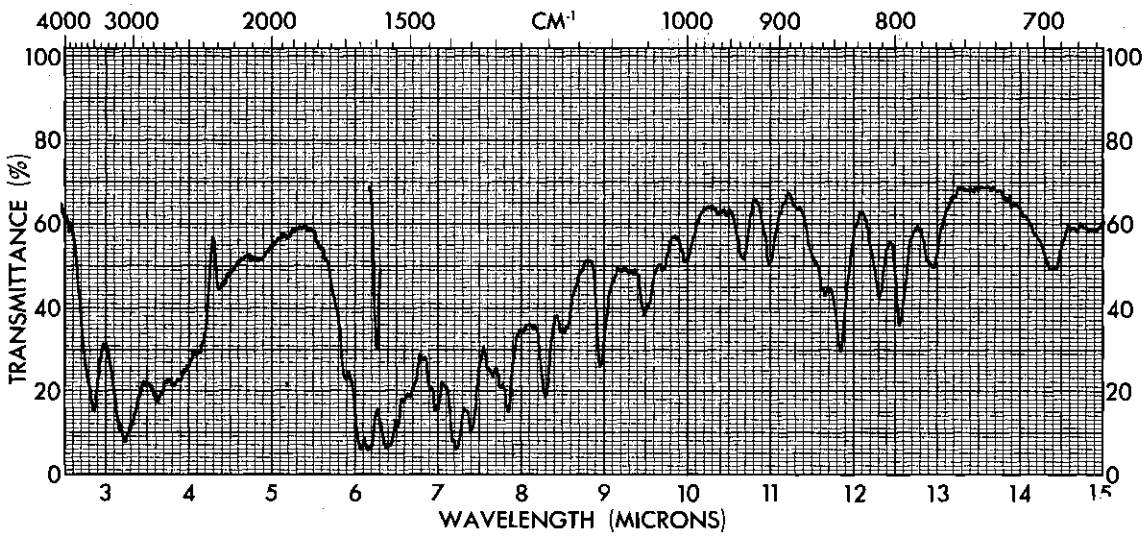

Figure 5. Infrared Spectrum of 4-Aminopyrrolidine-2,4-dicarboxylic Acid.

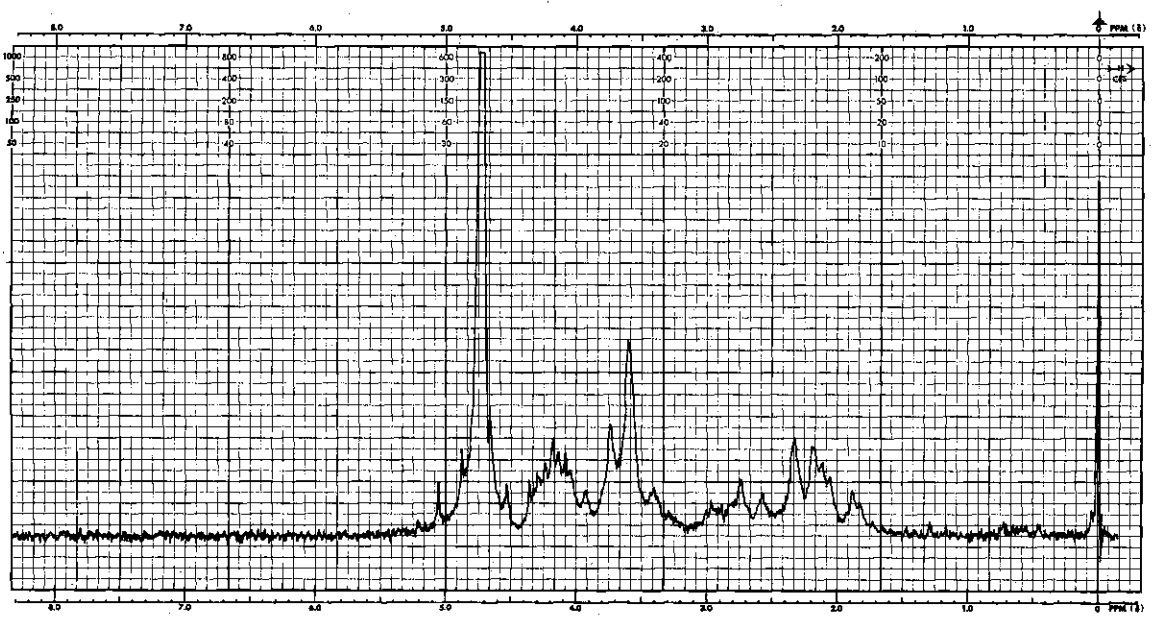

Figure 6. Nuclear Magnetic Resonance Spectrum of 4-Aminopyrrolidine-2,4-dicarboxylic Acid in Deuterium Oxide and Potassium Carbonate.

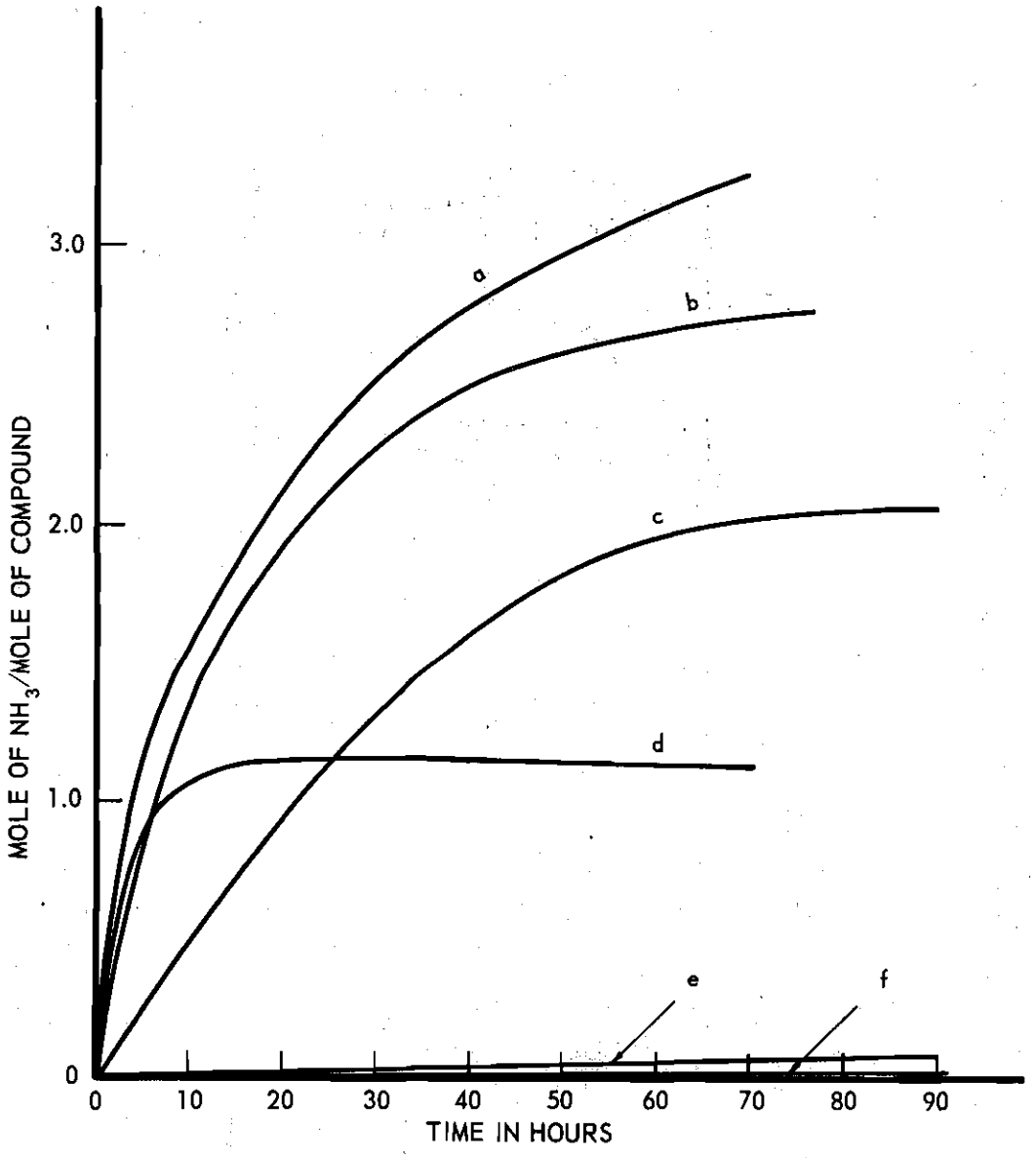

Figure 7. Moles of Ammonia liberated from the Barium Hydroxide Hydrolysis of Viomycin, Viomycidine, Urea, Serine, and α,β-Diaminopropionic Acid versus Time.

a. Barium Hydroxide Hydrolysis of Viomycin.

b. Barium Hydroxide Hydrolysis of Viomycidine.

c. Barium Hydroxide Hydrolysis of Urea.

d. Viomycin Curve minus Urea Curve.

e. Barium Hydroxide Hydrolysis of Serine.

f. Barium Hydroxide Hydrolysis of α,β-Diaminopropionic Acid.

VITA

Riyad Farid Nassar was born March 25, 1934, in Al-Monsif, Jubeil, Lebanon. He attended Al-Monsif elementary and secondary schools and was graduated from Aley High School, Aley, Lebanon in June, 1953.

In October, 1953 he entered the American University of Beirut, where he received the Bachelor of Science degree in June, 1957 and the Master of Science degree in chemistry in June, 1959. His research at the American University of Beirut was supported by a Research Corporation Fellowship.

In September, 1959 he enrolled in the Graduate Division of Georgia Institute of Technology where his research was mainly supported by a research assistantship sponsored by the National Institutes of Health. He was married on June 19, 1959 to Helen Ghusn and has two children, Nada and Ziad.